STUDIES ON PARASITISM AND ULTRASTRUCTURAL DEVELOPMENT OF *Choanephora cucurbitarum*

By

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DEDICATION

This dissertation is dedicated in loving memory of my father, Reverend Joe Agee, and to my sweet little girl, Kenya.

Darling Kenya,
If you can keep your head when life's temptations are bidding you to heed their beck and call,

If you can dream and have high expectations yet not mind working hard to win it all...

If you can take advice from those who love you, yet learn to make decisions for yourself

If you can cheer as others pass above you, and put your disappointments on the shelf...

If you can work and not make toil your master,

If you can gain and not make gold your aim,

If, whether you meet triumph or disaster, you can be proud of how you "played the game"...

If you can smile when odds are all against you, determined to try even harder still,

If when your foes have practically convinced you "you can't"--you buckle down and say "I WILL"...

If friends count more with you than wealth and power and you're at home alike with great and small,

If you can know success' finest hour yet place RESPECT and HONOR over all...

If you can be UNSELFISH and forgiving, and give thanks for the blessings you've received,

If you can face the challenge of living and, choosing right from wrong, are not deceived...

If you can put your heart into attaining the goal you seek, and do your best each day, yet, without hesitating or complaining, help others gladly as you go your way...

If you can live the Golden Rule each minute, and shape your course to GOD'S plan, yours is the World--and everything that's in it....And what is more, you'll REALLY be a WOMAN!

Love,

Mommy

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STUDIES ON PARASITISM AND ULTRASTRUCTURAL DEVELOPMENT OF *Choanephora cucurbitarum*

By

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August, 1978

Chairman: Dr. James W. Kimbrough
Co-Chairman: Dr. D. A. Roberts
Major Department: Plant Pathology

Choanephora cucurbitarum, the cause of blossom blight of squash and other cucurbits, is a member of the Choanephoraceae (Mucorales), a family of Zygomycetes characterized by producing sporangia, sporangiola, and zygospores. Striate, appendaged spores appear to be the most diagnostic feature of the family. The presence of various asexual reproductive spores and the role they play in the life cycle has led to considerable confusion in certain of the Choanephoraceae taxa. Much of the confusion has centered around the interpretation of whether the family produces unispored sporangiola or conidia. The literature reveals that there is still considerable confusion concerning taxonomic limits of various taxa in this group. The role that multispored sporangia, sporangioles, and zygospores play in the life cycle of *C. cucurbitarum* in Florida is as yet unanswered.

Studies on development and morphology using light microscopy, transmission and scanning electron microscopy have shown that C. cucurbitarum produces monosporous sporangiola, and not conidia. Sporangiospores are cleaved out in the sporangium by progressive cleavage. The appendages are curled in cross-section and consist of two electron-dense layers when viewed in longitudinal-section. Scanning observations of dehiscence show a suture along a preformed line on the spinulose sporangial surface. Sporangiospores are longitudinally striate and germinate laterally by one to two germ tubes.

This study marks the first time that light microscopy, transmission and scanning microscopy have been combined to observe zygospore development in the genus Choanephora. The dark, thick-walled zygospore is enclosed within a thin-walled, hyaline zygosporangial wall which sloughs off or cracks to reveal the striate nature of the zygospore. The zygospores are held on apposed, tong-like suspensors transversed by multiperforate septa.

Field and laboratory studies on parasitism show that C. cucurbitarum is a soil-borne pathogen. In Florida, the primary inoculum is derived from infested soil and secondary spread is effected primarily by bees (Apis spp) and spotted

cucumber beetles (Diabrotica punctata). Scanning electron microscopy observations failed to establish the spore-insect relationship, but spores were isolated readily from bees and beetles.

Infection is limited to the squash flower largely due to a chemotropic attraction for floral organs. Attempts were made to show anatomical relationships of spores to floral parts by use of scanning electron microscopy. Observation of these relationships made with light microscopy were not duplicated using scanning electron microscopy.

Zygospores and sporangiospores were not found associated with diseased plants from the field. A mating strain, "Atus," was isolated from morning-glory in Tuskegee Institute, Alabama. This strain mated successfully with all other isolates. Isolates of C. infundilifera, C. conjuncta, Blakeslea trispora and a possible new species of Blakeslea were collected during these studies.

GENERAL INTRODUCTION

Choanephora cucurbitarum (Berk. and Rav.) Thaxter is a common pathogen wherever squash and other susceptible crops are grown. Blossom blight, as the disease is referred to commonly on squash, causes serious economic losses in Florida. The fungus generally is regarded as a weak parasite or facultative parasite. Because of its prevalence in Florida, this investigation was undertaken to resolve areas of the fungus development and its disease cycle now in question.

Choanephora cucurbitarum is a member of the Zygomycotina. Members are classified according to the nature of their asexual spore type. The Choanephoraceae is a family within the order, Mucorales, which forms multispored sporangia. The large circinate sporangia are spiny and columellate. Spore release is not by deliquescence but by the splitting in half of the sporangia to release the sporangiospores in a slimy mass. Sporangiospores are dark, longitudinally striate with bipolar hyaline appendages. Dark, acolumellate sporangiola are formed by a blastosporic process similar to that found in many of the Fungi Imperfecti. A few plant pathologists and mycologists have

regarded these blastosporic spores as "conidia."

Chlamydospores are often formed on hyphae in older cultures. They are capable of germinating and functioning as asexual spores. The dark, thick walled zygospores are heterothallic and form on or buried within the substrate.

The following study consists of (1) a review of the current taxonomic status of the genera included within the family, Choanephoraceae; (2) ultrastructural studies of morphology and development of monosporous sporangiola; (3) ultrastructural studies on the germination of unispored sporangiola; (4) ultrastructural studies on multispored sporangia and sporangiospores; (5) ultrastructural studies on zygospore development; (6) ultrastructural studies on host-pathogen relationships; and (7) studies on the life-history of the pathogen before and after infection.

CHAPTER I

HISTORICAL REVIEW

Currey (1873) first described Choanephora as a species of Cunninghamia and designated it as the type Cunninghamia infundilifera on flowers of Hibiscus rosae-sinensis from Calcutta, India. He did not hesitate in placing this new and exciting fungus in the Fungi Imperfecti (Mucedines) after observing the conidial like fructifications on diseased tissues. Later, learning that a higher plant had already been given the same generic name, Currey (1873) renamed his isolate Choanephora cunninghamia. Saccardo (1891) gave the type its present name, Choanephora infundilifera.

Six years after Currey's original description, Cunningham (1878) observed that Currey's new fungus, in addition to conidia, produced Mucoralean type zygospores, sporangia and chlamydospores. Dr. Cunningham contributed the original drawing upon which Currey made his 1873 observations. He concluded that Currey's Mucedinous fungus was indeed a member of the Mucorini. Brefeld (1872) divided

the Mucorini into two subfamilies, one which produces sporangia and the other conidia. Van Tieghem and Le Monnier (1873) refuted the idea of conidial states in the Mucorini. They suggested that the structures were monosporous sporangia or aerial chlamydospores.

During the month of September in 1894, Cunningham (1895) observed a destructive blight of Ipomoea rubro-caerulea, Hook. He subsequently isolated and described the causal agent. Thus, a second species was established and named Choanephora simsonii. He reported that the conidiophores were smaller than those of C. infundilifera, the sporangiospores were appendaged on each end, funnels were absent, and the zygospores were described as different from C. infundilifera. Zycha (1935) indicated that C. simsonii was synonomous with C. infundilifera. Hesseltine (1953) and Poitras (1955) expressed doubts as to the validity of this species but retained it in their treatments. Milko and Belyakova (1969) concluded that the two species were synonymous.

Rhopalomyces cucurbitarum was described by Berkley and Ravenel, after it had been isolated from decayed squash in South Carolina (Berkley, 1875). Mucor cucurbitarum was described by the same authors in 1875. Marchal (1893) concluded that R. cucurbitarum (Berk. and Rav.) was a

variety of Rhopalomyces elegans which was described by Corda (1839). He suggested that R. cucurbitarum (Berk. and Rav.) be renamed, Rhopalomyces elegans var. cucurbitarum (Berk. and Rav) Corda.

Thaxter (1903) observed what he called the first American Choanephora. He later learned through a literature search that a similar fungus had already been described by Berkley (1875). While visiting Eustis, Florida, Dr. Thaxter obtained isolates on flowers of cultivated Hibiscus and wild malvaceous plants which proved to be identical to his New England isolate. After observing several specimens he concluded that his isolate was the conidial stage of Choanephora. He further suggested that this isolate was synonymous with R. cucurbitarum, Mucor cucurbitarum and Aspergillus cucurbitaceous. He named the New England isolate Choanephora cucurbitarum (Berk.and Rav.) Thaxter. Moeller's (1901) C. americana was declared synonymous to C. cucurbitarum by Thaxter in the same publication.

Vuillemin (1904) reported that R. cucurbitarum was related more closely to the genus Rhopalomyces than Choanephora as Thaxter (1903) suggested. He proposed that the fungus be renamed Choanephorella cucurbitarum. Zycha (1935) concluded that this organism was synonymous to C. cucurbitarum.

Lendner (1908) included Choanephora, Cunninghamella and Chaetocladium in the family Chaetocladiaceae. Fitzpatrick (1930) combined Choanephora, Cunninghamella, Sigmoideomyces and Thamnocephalis in the family Cunninghamellaceae. Naumov (1939) transferred Cunninghamella, Sigmoideomyces and Thamnocephalis to the newly erected family, Cunninghamellaceae. Cokeromyces was transferred to the Thamnidiaceae from the Choanephoraceae by Poitras (1955). The genus had been placed originally in the Choanephoraceae by Shanor et al. (1950).

Cunninghamella mandshuria was described by Saito and Naganishi (1915) but Tai (1934) transferred it to the genus Choanephora as C. "manshuria." Zycha (1935) and Hesseltine (1953) concluded that C. manshuria was synonomous to C. cucurbitarum.

Thaxter (1914) established the genus Blakeslea for a species that was very similar to Choanephora but produced sporangioles instead of conidia. Blakeslea was isolated by Thaxter from a Botrytis culture obtained from cowpea in Gainesville, Florida. The branching of the hyphae and the presence of successive constrictions on the hyphal branches suggested that this species was different from Choanephora. Thaxter stated that the conidia of Choanephora should be

called monosporous sporangiola and he tried to demonstrate an outer sporangial wall but failed.

Sinha (1940) after observing a wet rot of Colocasia due to secondary infection by Choanephora suggested the production of few-spored sporangiola was a nutritional phenomenon. He concluded that Blakeslea was not a valid genus after observing conidia in cultures of B. trispora and suggested the name be changed to Choanephora trispora.

Choanephora dicotoma on leaves of tobacco was described by Gandrup (1923) but Zycha regarded this species as synonymous to Blakeslea trispora. Poitras (1955) concluded that Blakeslea and Choanephora were synonymous. Hesseltine (1957) supported Poitras's treatment of the two genera as synonymous.

Since 1955 three additional species of Blakeslea have been described. Blakeslea circinans was described by Naganishi and Kawakami (1955). Hesseltine (1957) transferred B. circinans to Choanephora after observing the zygospores. Mehrotra (1963a) described B. tandonii and later recognized three species: Blakeslea trispora, B. circinans and B. tandonii (Mehrotra, 1963b). Mehrotra and Bayal (1968) described B. monosporus and concluded that on the characters of this species the genus Blakeslea should be retained as a valid genus.

Couch (1925) described a new dioecious species of Choanephora which he named Choanephora conjuncta. In his description Couch separated C. conjuncta on the following characters: (1) wall striations on the sporangiospores, (2) different zygospore hyphal systems, (3) punctate rather than striate "conidia," and (4) funnel formation. Dantas (1953) observed C. conjuncta outside the United States for the first time and regarded C. conjuncta and C. infundilifera as synonymous. Poitras (1955) after observing herbarium specimens of C. conjuncta reported that it was synonymous to C. cucurbitarum. Hesseltine (1957), in contrast to Poitras, retained the species. After examining isolates from soil provided by Dr. Julian Miller at the University of North Carolina, he stated that his isolates were identical to those described by Couch (1925). The treatments of Cheng (1964), Mehrotra and Mehrotra (1964), and Mil'ko and Belyakova (1969) have retained C. conjuncta as a valid species.

During the same year in which Couch described C. conjuncta, Eddy (1925) described C. persicaria as causal agent of storage rot of peaches. Eddy (1925) stated that his new species produced only linearly striate, appendaged sporangiospores. Dantas (1953) regarded C. persicaria as a doubtful species. Hesseltine (1960) obtained type

isolates of Eddy's species from the Roland Thaxter herbarium. He found living material in the Centraalbureau voor Schimmelcultures, Baarn Holland,and observed that these isolates were identical to the type material; pathogenicity studies were positive on inoculated peaches. Hesseltine (1960) transferred Eddy's species to a new genus, Gilbertella, which was named in honor of Professor E. M. Gilbert.

Gilbertella persicaria, according to Hesseltine, represents a link between the Mucoraceae and Choanephoraceae. He stated that the Mucoraceae should be expanded to include species with warty, parallel zygospores and appendaged sporangiospores. Mehrotra and Mehrotra (1964) agreed with Hesseltine's classifications and conclusions. Cheng (1969) stated that G. persicaria should not be transferred to the Mucoraceae. According to Cheng (1964) the Choanephoraceae developed from Rhizopus. He supports his hypothesis by saying that Rhizopus and fungi in the Choanephoraceae cause similar type diseases on higher plants and have common spore characters. He further stated that Gilbertella is an intermediate between the two families and should be retained in the Choanephoraceae.

Mehrotra and Mehrotra (1961) described Choanephora heterospora (Mehrotra and Mehrotra, sp. nov.) on a dead

insect. When cultured under poor nutritional conditions, this fungus produces heterosporous sporangioles in the same sporangiolum. Appendages were produced at several locations on the spore surface of the larger spores and on both ends of the smaller spores. Several germ tubes were produced upon germination of the spore.

In the past there was a tendency to place Zygomycetes which produced blastosporic type spores on sporangioliferous heads in the Choanephoraceae. Poitras (1955) did not agree with such a strict character and removed Cokeromyces from the Choanephoraecae to the Thamnidiaceae.

The taxonomy of the Choanephoraceae remains in controversy. The general consensus is that Choanephora and Blakeslea are synonymous and the genus Gilbertella is a member of the Choanephoraceae.

Chienand Wu (1977) concluded that C. infundilifera, C. conjuncta and C. cucurbitarum should be classified as a single variable species. They suggested the accepted form should be C. cucurbitarum, f. cucurbitarum, C. cucurbitarum f. conjuncta and C. cucurbitarum, f. infundilifera. The authors accepted C. circinans as a valid species since it failed to mate with any of the above isolates. The fate of Choanephora trispora was not explained in their report.

CHAPTER II

STUDIES ON THE MORPHOLOGY AND DEVELOPMENT OF MONOSPOROUS SPORANGIOLA IN *Choanephora cucurbitarum*

For more than a century there has been a controversy as to whether certain members of the Mucorales such as *Choanephora* and its allies produce true conidia. Van Tieghem and Le Monnier (1873) suggested that conidia and sporangia could be distinguished according to the manner of germination. They demonstrated that when pressure is applied to the sporangia the spore contents escape partially or completely. Cunningham (1878) argued against such a distinction, stating that certain conidia could germinate by at least partial escape of the spore contents. Reports of this form of conidial germination in the Mucorini were never documented by Cunningham.

Mangin (1899) concluded that the conidia of *Piptocephalis monosporus* were not conidia but unispored sporangioles. Benjamin (1959, 1966) described the Kickxellaceae in which the genus *Piptocephalis* was placed as being one to two spored.

Thaxter (1914) reported that the sporangia of Haplosporangium decipiens Thaxter produced one to two spored merosporangia. Members of the family Mortierellaceae are now described as possessing unispored and multispored sporangia.

Torrey (1921) compared the spores of Cunninghamella spp. to the conidia of higher fungi, and stated that he was unable to repeat the observations of Bainier and Sartory (1913). The latter authors had concluded from their observations that conidia similar to higher fungi were produced by Cunninghamella spp. Torrey (1921) stated that developmentally these spores were related more closely to monosporous sporangiola.

Poitras (1955) suggested that Sigmoideomyces Thaxter and Thamnocephalis Blakeslee may be the only conidial forms in the Mucorales. He was doubtful that these two members of the Cunninghamellaceae were true Mucoralean genera. Samson (1969) and Hesseltine and Ellis (1973) placed both genera in the Cunninghamellaceae.

Thaxter (1914) suggested that Choanephora produces monosporous sporangiola. He tried separating the sporangial wall from the spore wall but was unable to separate the two walls mechanically. He concluded that Blakeslea produces few-spored sporangiola and Choanephora produces conidia.

Sinha (1940) combined Blakeslea and Choanephora after he induced unispored sporangia in Blakeslea. Poitras (1955) supported Sinha's conclusion when he reported observing

separation of the spore and sporangial walls in Choanephora. Benjamin (1959) suggested that these blastosporic propagules were unispored sporangiola, that is, a single spore is formed when protoplast rounds up and becomes surrounded by a spore wall and an outer sporangial wall. Kendrick (1971) defines a conidium as "a specialized, non-motile, asexual propagule, usually caduous, not developing by cytoplasmic cleavage or free-cell formation"; however, this definition also describes the monosporous sporangiola produced by certain species of mucoraceous fungi. Concerning the Choanephoraceae, Hesseltine and Ellis (1973) stated ". . .in addition to sporangia, all but two species possess distinct fruiting stalks which bear conidia (one-spored sporangiola) or sporangiola."

Hawker et al. (1970) and Dykstra (1974) reported that their ultrastructural observations revealed that Cunninghamella spp. produce conidia. Khan and Talbot (1975) have shown that Mycotypha microspora and Cunninghamella echinulata produce monosporous sporangiola, not conidia.

Although many investigators have argued against the existence of true conidia in the Choanephoraceae, conclusive evidence is still lacking. The objective of this study is to present morphological and developmental evidence that unispored sporangiola and not conidia are produced by the Choanephoraceae.

Materials and Methods

Collection and Isolation

Isolates used in light microscopy studies were taken from diseased squash on the student agricultural farm at the University of Florida in the spring of 1978. Specimens for electron microscopy were isolated from diseased squash in a local garden in 1975 in Gainesville, Florida. Specimens for scanning electron microscopy were collected and spores were isolated from diseased squash in the fall of 1977. Specimens were taken from plots at the University of Florida horticultural unit. All specimens were placed in a styrofoam container and delivered to the laboratory for further observation.

Spore heads were taken with a flamed transfer needle and placed on various types of media. Media used included Emerson YpSs (YPS), 1/2 cornmeal (CM), potato dextrose agar (PDA), squash agar and petal decoction agar. All of the above are commercial grade media except squash agar and petal decoction.

Squash Agar

Squash baby food	64 grams
Agar	10 grams
Distilled water	500 milliliters

The medium was autoclaved for 15 min and upon cooling streptomycin and penicillin were added.

Squash Petal Decoction

Squash petals	25 grams
Agar	10 grams
Distilled water	500 milliliters

Twenty-five grams of petals were placed in 500 ml distilled water and boiled for one hour. After boiling, the liquid was filtered and petals discarded. The remaining liquid was made up to 500 ml with distilled water, 10 g of agar were added, and the medium was autoclaved for 15 min. To reduce bacterial growth, streptomycin and penicillin were added to agar that was cooled to 45 C.

All cultures were incubated at room temperature. Hyphal tips were taken from cultures and incubated at 25 C or left at room temperature.

Light Microscopy

Spore heads were selected at various stages of maturity from cream to dark brown. Selected stages were mounted in water, lactophenol cotton blue or phloxine-KOH for observation under light and phase microscopy.

Electron Microscopy

Thalli of monosporous sporangiola were placed on a thin layer of water agar and overlaid with another layer of water agar. Small blocks ($1mm^2$) were cut and placed in buffered (pH7.2) Karnovsky's (1965) fixative (2% glutaraldehyde and 2% paraformaldehyde) for 15 min at room temperature followed by 105 min at 4 C. After fixation blocks were washed in 0.1M sodium cacodylate, pH7.25. Blocks were post-fixed in 1% osmium tetroxide overnight at 4 C. After osmium fixation, blocks were again washed several times in 0.1M buffer. Specimens were dehydrated through a graded ethanol series (25, 50, 75, 95, 100). Ethanol was replaced by placing blocks in two changes of 100% acetone. After passing through an acetone-plastic series, blocks were embedded in 100% Spurrs (1969) embedding medium. Embedded specimens were polymerized for 24 hours in a 60 C oven.

Ultra-thin sections were cut on a Sorvall MT-2 ultramicrotome. Sections were placed on 300 mesh grids and post-stained for 15 min in uranyl acetate and 5 min in lead citrate. Specimens were observed with a Hitachi HU-11E electron microscope.

Scanning Microscopy

Monosporous sporangiola were placed on millipore filters and fixed for 2 hours in Karnovsky's (1965) fixative. After fixation the fixative was drawn off and replaced with a graded ethanol series (25-100%) and acetone (100%). Specimens were critical point dried and coated with gold in a Hummer-II sputter coater and observed on a Zeiss scanning electron microscope.

Results

Development

Squash agar and petal decoction agar were superior to all other media tested for the production of the unispored form of Choanephora. Much more mycelial growth was produced on commercial media.

After maturation of the sporangiophore, several branchlets (Fig. 1) begin to radiate out from the distally swollen (Figs. 2, 11, 12) sporangiophore or primary vesicle. The branchlets begin to swell and blow out at their distal ends (Fig. 2). Swelling continues until the young ampulla or secondary vesicle is formed (Figs. 3, 30). The branchlets become subdichotomously branched (Fig. 3)

as the thallus continues to mature. A small swollen area (Figs. 4, 31) appears on the ampulla wall. Swelling appears to be synchronous over all ampullæ (Fig. 6). The areas continue to swell (Fig. 5) until stalks (denticels) can be observed supporting what become the young sporangiola (Fig. 7). The sporangiolum's outer wall appears dark and the spore content granular (Fig. 8). The spores are cream colored during the early stages of development, but quickly turn dark brown at maturity. The striate nature of the sporangiola can be seen at this time (Fig. 9). When the sporangiolum is detached from the denticel a pedicel is formed at the base of the sporangiolum (Fig. 9). The unispored nature of the spore can be seen as the spore content exudes from the sporangiolar walls (Fig. 10). The branchlets do not show septation at the sporangiophore or the ampulla (Fig. 11). When the branchlets are separated from the primary vesicle, distinct tears are left at the points of detachment (Fig. 12).

Electron Microscopy

Ultrastructural observations confirm that Choanephora cucurbitarum produces monosporous sporangiola and not conidia. The spores develop on secondary vesicles termed

ampullae (Figs. 4, 30). The ampulla contains nuclei, mitochondria, ribosomes and lipid bodies (Figs. 13, 16). As the ampulla matures it becomes vacuolated (Fig. 16). The mature ampulla has two distinct wall layers (Figs. 13, 14), a thin outer electron-dense layer and a thicker, lighter layer next to the plasmalemma.

Denticels appear to develop blastosporically on the ampulla wall (Fig. 31). The protrusion continues until the denticel is fully developed (Fig. 15). The outer and inner wall layers of the denticel are continuous with the inner and outer wall of the ampulla (Figs. 20, 31, 32). The denticel continues to elongate until the young sporangiolum develops (Figs. 18, 32). The denticel's cytoplasm contains many of the organelles seen in the ampulla (Fig. 15) since the contents of the latter pass into the denticel during blow out and maturation (Figs. 15, 16, 18). The vacuolar contents become osmiophilic (Fig. 17) in the mature denticel.

The young sporangiolum is surrounded by a thin sporangiolar wall which is continuous with the denticel and ampulla outer wall (Figs. 17, 18, 20, 32). The young spore contains nuclei, mitochondria, ribosomes, endoplasmic reticulum, lipid bodies and a light staining material

(Figs. 19, 27, 28). The fat reserves in the mature spore disappear during maturation and germination (Fig. 29). Few vacuoles are present in the sporangiola at this stage of development; however, the fully matured spore becomes vacuolated and osmiophilic areas appear inside the vacuoles (Fig. 29).

The two-layered spore wall forms around the cytoplasm (Figs. 17, 19, 32). The spore wall and sporangiolar wall remain distinct during their development (Fig. 19). During the early stages of development the sporangiolar wall develops a spinulose surface. The spines are dome shaped and stain lighter than the sporangiolar wall (Fig. 19). The outer spore wall becomes very electron dense and irregularly thickened as it continues to develop (Figs. 19, 20, 30) and the lighter inner spore wall becomes much wider than the outer spore wall (Figs. 26, 33).

The spore wall continues to thicken until it cuts the spore cytoplasm off from that of the denticel (Fig. 20). When the sporangiolum is detached from the denticel, a pedicel is left on the sporangiolum at the point of detachment (Fig. 25). If the denticel is empty (Fig. 25), the break produces a hollow pedicel.

Scanning Electron Microscopy

The mature thallus has several spore covered ampulla which develop on branchlets derived from the distally swollen sporangiophore (Fig. 21).

Sporangiola arise from denticels on the ampulla wall (Fig. 22). The spores are longitudinally striate and, when detached leave part of the denticel (Figs. 23, 24) attached to the ampulla.

Discussion

The monosporous sporangiola of Choanephora cucurbitarum (Berk and Rav.) Thaxter develop botryoblastospores on the surface of secondary vesicles or ampullae. Barnett and Hunter (1972) define botryoblastospores as blastospore type conidia which are produced on well-differentiated swollen cells. Many spores are borne simultaneously and form clusters or beads, solitarily or simply on branched acropetalous chains; mature condia are easily deciduous revealing minute denticels on the spore-bearing cells. Cook (1977) and Khan and Talbot (1975) reported that Cunninghamella echinulata Thaxter, a member of the Cunninghamellaceae, produces conidia-like blastospores.

Several conidial fungi in the Deuteromycetes follow developmental patterns strikingly similar to the Mucor form described by the authors above (Hughes and Bisalputra, 1970; Cole, 1973; and Cook, 1974).

The ampulla of C. cucurbitarum is surrounded by an outer and inner wall. The outer and inner walls of the ampullae are continuous with the outer and inner walls of the denticel. New wall layers are not formed beneath the ampulla or denticel at blow-out. Cook (1977) reported that in Oedocephalum roseum Cook, a true conidial fungus, a new wall layer is formed at blow-out but the ampulla and denticel of C. enchinulata, a fungus with monosporous sporangiola, remains two-layered throughout development.

Cook (1977) reported that a septum is formed after the ampulla of Oedocephalum roseum reaches maturity. The septum is located between the ampulla and emerging denticel. Khan and Talbot (1975) stated that septa are not formed in C. echinulata but a septum is formed in the sister genus, Mycotypha microspora Fenner. The septum formed in Mycotypha differs from that of true conidia in that the septum does not form part of the spore wall. They suggested that septal characters distinguish true conidia from monosporous sporangiola. My observations on septa

in this report are consistent with those of Khan and Talbot (1975) in C. echinulata.

Khan and Talbot (1975) reported that in C. echinulata the spore wall and sporangiolar wall are applied closely and appear fused. Khan (1975) states that the sporangiolar wall of C. echinulata can be seen as it sheds during germination. Hawker et al. (1970) and Dykstra (1974) failed to recognize the sporangiolar wall as being separate from the spore wall in the species of Cunninghamella. Khan and Talbot (1975) reported that they observed that Mycotypha differs slightly from C. echinulata in that the sporangiolar wall does not shed during maturation. Failure to recognize the fact that a single sporangiospore is surrounded by a single sporangiolar wall has led to much of the controversy over the existence of true conidia in the Mucorales (Cunningham, 1878; Thaxter, 1914; Hesseltine, 1957; Hawker et al., 1970; and Dykstra, 1974).

Alexopoulos (1962) explains that mycologists generally refer to sporangia in the Peronosporales, whereas, plant pathologists retain the term conidia for these deciduous structures. He suggests that it makes little difference whether such structures are called sporangia or conidia as long as there is an understanding of function and development.

Evidence presented by Khan and Talbot (1975) and in this study show that there are developmental differences between conidia and monosporous sporangiola and the two germs should not be used interchangeably.

The developmental differences between conidia and sporangiola can be summarized as follows: (a) the ampullae and denticels of monosporous sporangiola remain two-layered throughout maturation whereas in conidia new wall layers are formed; (b) no septa are formed between the ampullae and denticels or between the denticels and spores in sporangiola, while in conidia septa are formed to delimit the ampullae from the denticels and to delimit the conidial initials from the denticels; and (c) the outer wall of the sporangiolum is distinct from the two-layered spore wall, thus forming the sporangiolar wall, while in the conidial initial the walls remain fused.

Chapter II

Figures 1-3. Development of ampulla of Choanephora.

Figure 1. Development of branches from main sporangiolophore. X400

Figure 2. Branch swells to produce young ampulla. X400

Figure 3. Fully developed ampullae. X250

Figures 4-10. Development of unispored sporangiola.

Figure 4. Blow-out of denticel. X600

Figure 5. Young unispored sporangiola on ampulla. X1000

Figure 6. Sychronous development of spores on ampulla. X200

Figure 7. Denticels complete and sporangiola attached. X650

Figure 8. Mature sporangiola. X500

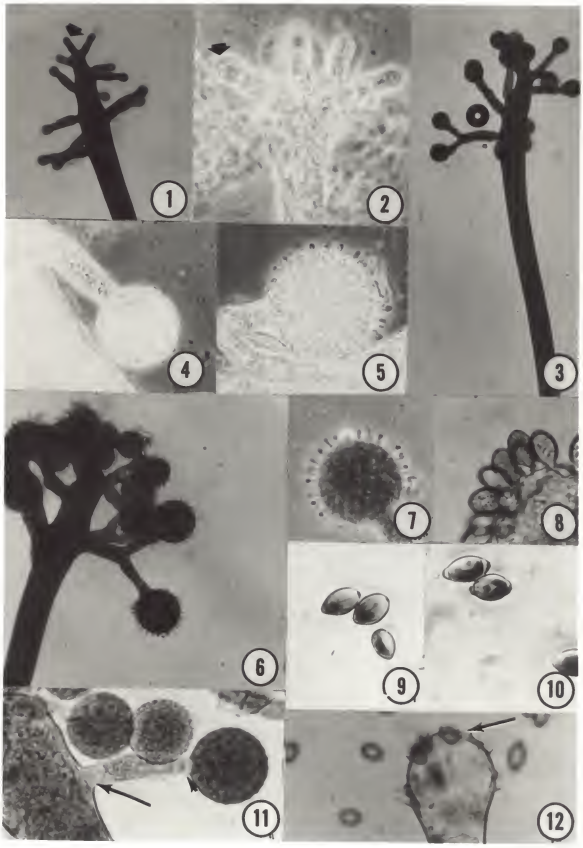
Figure 9. Pedicel and striate sporangiolum. X500

Figure 10. Sporangiospore exuding from sporangiolum. X500

Figure 11-12. Sporangiophore and primary vesicle with aseptate branch and ampulla.

Figure 11. Aseptate branch and ampulla. X600

Figure 12. Primary vesicle after branchlets are torn away. X600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Chapter II

Figures 13-20. Ultrastructural aspects of sporangiolum development of Choanephora cucurbitarum.

Figure 13. Ampulla with two-layered wall. Inner layer (IL). Outer layer (OL). Mitochondria (M). Vacuole (Vo). X72,000

Figure 14. Ampulla wall adjacent to denticel. X72,000

Figure 15. Denticel arising from ampulla. Lipid (L). X36,000

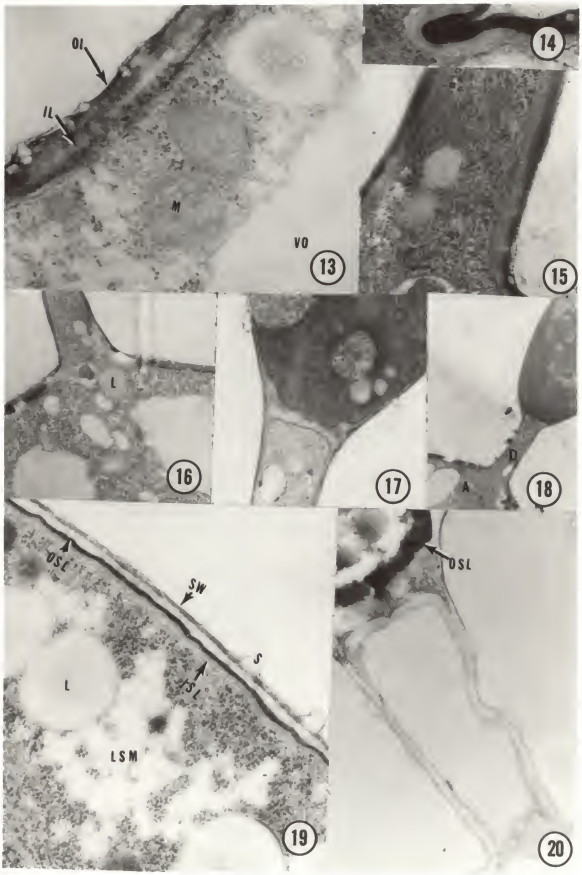
Figure 16. Denticel and ampulla. X12,000

Figure 17. Unispored sporangiolum attached to denticel. X16,000

Figure 18. Ampulla (A). Denticel (D). Sporangiolum. X6,300

Figure 19. Ampulla, outer sporangiospore wall developing. Light staining material (LSM). Lipid (L). Spines (S). Spore wall (SW). Inner spore layer (ISL). Outer spore layer (OSL). X64,000

Figure 20. Sporangiospore outer layer has separated denticel and sporangiolum. Outer spore layer (OSL). X30,000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Chapter II

Figures 21-24. Scanning microscopy of Choanephora cucurbitarum.

Figure 21. Mature sporangiophore with sporangiola. X3,000

Figure 22. Ampulla arising from sporangiophore branchlet. X4,000

Figure 23. Denticel and sporangiola attached to ampulla. X4,000

Figure 24. Broken denticels after sporangiola release. X4,000

Figures 25-29. Ultrastructure of mature sporangiolum after release from ampulla.

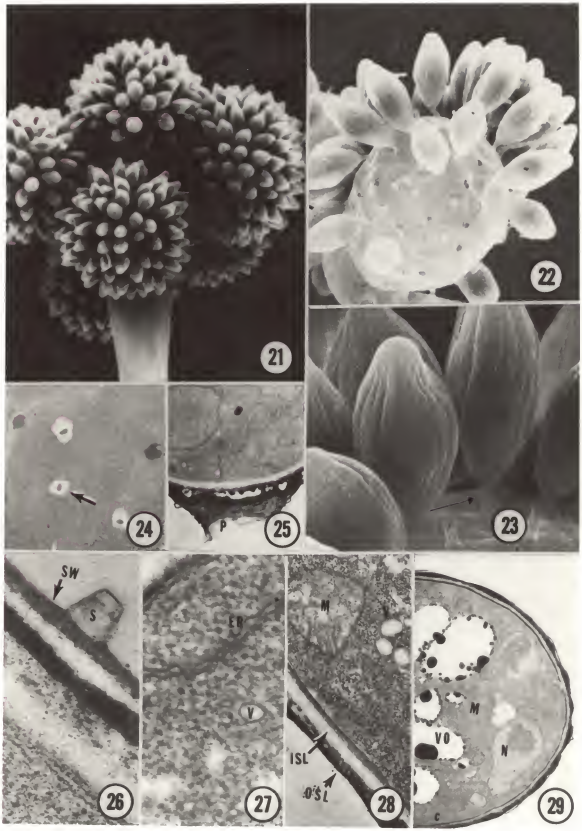
Figure 25. Pedicel (P) with attached sporangiolum. X12,000

Figure 26. Sporangial and sporangiospore walls. Spines (S). Spore walls (SW). X39,000

Figure 27. Endoplasmic reticulum (ER). Vesicles (V). Light staining material (LSM). X39,000

Figure 28. Unispored sporangiolum after shedding sporangial wall. Inner spore layer (ISL). Outer spore layer (OSL). Vesicles (V). Mitochondria (M). X10,000

Figure 29. Multinucleate condition of unispored sporangiolum wall shedding. Vacuole (Vo). Mitochondria (M). Nuclei (N). X7,800.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Chapter II

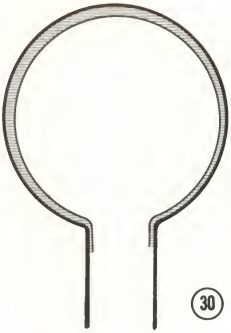
Figures 30-33. Schematic representation of sequence and origin of wall layers surrounding the unispored sporangiola of Choanephora cucurbitarum.

Figure 30. Two-layered ampulla wall.

Figure 31. Blow out of denticel with two-layered wall continuous with ampulla wall.

Figure 32. Sporangiolar wall and single sporangiospore wall. Spines have developed on sporangiolar wall.

Figure 33. Two-layered sporangiospore wall enclosed in sporangiolar wall.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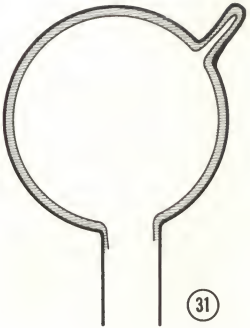
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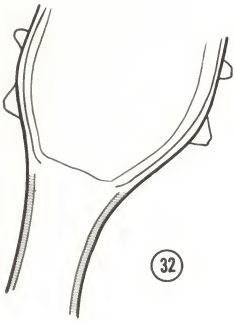
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CHAPTER III

GERMINATION OF MONOSPOROUS SPORANGIOLA OF *Choanephora cucurbitarum*

Brefeld (1872) established two subfamilies in the Mucorini. He stated that they either produce sporangia or conidia. Van Tieghem and Le Monnier (1873) concluded that conidia and sporangia could be separated according to the manner in which each germinates. They reported that the sporangium of *Chaetocladium jonesii* contained a single spore and that the germination of this species and conidial species differed.

Cunningham (1878) questioned the credibility of the observations presented by Van Tieghem and Le Monnier (1873). He reported that the genus *Choanephora* produces both conidia and sporangia and that the germination of conidia and sporangiospores could occur by the same process. Cunningham was the first investigator to formally ask the question, how are monosporous sporangia to be distinguished from conidia?

Bartnicki-Garcia (1968) summarized the role of fungal cell walls in the systematics and morphogenesis of

fungi. He reported that vegetative wall formation during germination occurs by three mechanisms or types. Each type, according to Bartnicki-Garcia, is characteristic of a certain group of fungi.

Type I is characteristic of the conidia of higher fungi. The vegetative wall is derived as a direct extension of the spore wall. Hawker and Hendy (1963) have observed this type in Botrytis cinerea. Type II is the de novo formation of wall around naked protoplast as in the zoospores of the aquatic phycomycetes. In Type III the vegetative wall is formed de novo under the spore wall. Hawker and Abbott (1963) were the first investigators to report this mechanism in the sporangiospores of Rhizopus spp. Hawker (1966) and Bracker (1966) have concluded that the same mechanism exists in germinating sporangiospores of Cunninghamella elegans Lendner and Gilbertella persicaria (Eddy) Hesseltine, respectively.

Hawker et al. (1970) reported that in Cunninghamella elegans the innermost layer of the spore wall is changed chemically and becomes the wall of the emerging germ tube. Dykstra (1974) concluded that Cunninghamella vesiculosa Misra does not form a new wall prior to or during germination. He suggested that the inner spore wall surrounds

the germ tube and without being changed chemically becomes the wall of the germ tube. In the multispored form, Hesseltinella vesiculosa Upadhyay, he observed the de novo formation of the germ tube wall and stated that it was continuous with the innermost wall layer.

Khan (1975) concluded that the walls of Cunninghamella echinulata are not changed chemically as suggested by Hawker (1970). He stated that Dykstra's conclusion on Cunninghamella vesiculosa was not valid since his observations were taken too late in the formation of the germ tube to make a valid conclusion. Khan (1975) reported on a germination mechanism in Cunninghamella echinulata which was different from that observed by other investigators. He concluded that the germ tube wall was different from any of the pre-existing wall layers before the onset of germination.

The following study of Choanephora cucurbitarum reports on the germination mechanism in unispored sporangiola of this species.

Materials and Methods

Specimens of diseased squash were brought into the laboratory from a local garden in the summer of 1975.

Spore heads were taken on a flamed needle and placed on Emerson's YpSs agar (YPS) and left at room temperature. After a luxuriant growth of the fungus was produced in culture, spores were prepared for further observations.

Spores for electron microscopy were prepared according to the procedure described by Dykstra (1974). Seven-day old cultures were flooded with a spore germination medium prepared as follows: casein, 2 gms; dextrose, 10 gms; K_2KPO_4, 1.0 gm; $MgSO_4 \cdot 7H_2O$, 0.5 gms; distilled water, 1 liter.

After flooding with the germination medium the surface of the plate was rubbed briskly with a glass rod to remove the hyphae and spores from the agar surface. The resulting slurry was aseptically filtered through a copper wire mesh to retain the hyphae and allow spores to pass through.

Ten milliliters of the slurry was decanted into a 50 ml flask and incubated on a rotary shaker at 110 rpm at room temperature. Spores were removed at 15 min intervals up to 90 min. At the preferred stage of germination (45 min) the flask contents and Karnovsky's (1965) fixative, buffered with sodium cacodylate at pH 7.0, were mixed at a ratio of 1:1 v/v.

The suspension was centrifuged at 800 rpm for 2 min, the supernatant removed and half-strength Karnovsky's added. After 15 min at room temperature and 45 min at 4 C the suspension was centrifuged until a pellet was formed. The pellet was washed with 0.05m sodium cacodylate for 10 min, buffer removed and replaced with 1.5% water agar at 50 C. The mixture was stirred and centrifuged at 1500 rpm for 4 min. The pellets were cut into small pieces, rinsed twice in sodium cacodylate buffer for 20 min and post-fixed in 1% osmium tetroxide overnight at 4 C.

After post-fixation the pellets were dehydrated through a graded alcohol series and acetone. Specimens were infiltrated with plastic and embedded in Spurr's (1969) embedding media. Blocks were polymerized in a 60 C oven over night.

Ultrathin sections were cut on a Sovall MT-2 ultramicrotome. Sections were placed on formvar coated grids and post stained in 2% uranyl acetate for 15 min and lead citrate for 5 min. Further observations were made on a Hitachi HU-11E electron microscope.

Scanning Microscopy
Electron

Spores were scraped from the surface of culture plates by flooding with distilled water and rubbing briskly with a glass rod. A suspension of the slurry was removed with a sterile pipette and placed on sliced squash fruit. Tissues were incubated for 90 min at room temperature. After this time the fruit tissues with germinating spores were fixed in buffered Karnovsky's fixative (1965) and post-fixed in 1% osmium tetroxide overnight at 4 C. The tissue with spores was washed in sodium cacodylate buffer and water 1:1 v/v and dehydrated through a graded alcohol and acetone series.

Specimens were critical point dried and coated with gold in a Hummer-II sputter coater. Scanning observations were made with a Zeiss scanning electron microscope.

Light Microscopy

A small drop of the suspension from the germination medium was placed on a slide, cover-slipped and observed under phase and light microscopy at intervals of 15 min up to 90 min.

Results

Germination in unispored sporangiola is accompanied by swelling prior to germ tube emergence (Fig. 34). Germ tubes emerge laterally on germinating spores (Figs. 34, 35) producing usually a single germ tube.

In the quiescent spore, the outer spore wall appears deeply furrowed and convoluted (Fig. 36). The organelles, though barely distinguishable, include nuclei, mitochondria, and numerous lipid bodies (Figs. 36, 37). As germination proceeds the sporangiolar wall sheds (Fig.37) and the surface of the sporangiolum becomes more spherical (Figs. 37, 38). The organelles become more visible as the spores become more amenable to biological staining (Fig. 38).

Prior to germ tube emergence the sporangiospore has a furrowed, electron-dense outer wall and an electron-translucent inner wall (Figs. 36, 39). The germ tube continues to emerge, displacing the spore wall (Fig. 39). If the sporangiolar wall has not shed, it cracks at several sites (Fig. 38). The spore wall remains morphologically the same during this period (Fig. 48).

A new vegetative layer forms over the incipient germ tube (Figs. 39, 40, 43). This layer becomes progressively

thinner and fades a short distance behind the emerging germ tube (Fig. 42). The vegetative layer does not surround the entire spore as reported in Bartnicki-Garcia's (1968) Type III. The new vegetative layer and the wall of the germ tube are continuous and distinct from any of the pre-existing walls (Fig. 45). Remnants of the inner spore wall could be seen on the new vegetative layer (Fig. 44).

Germination and swelling brought about the appearance, change and loss of some cellular organelles. Vesicles were common at the site of germ tube emergence and wall formation (Figs. 44, 46,50). The amount of endoplasmic reticulum and ribosomes increased during germination (Fig. 46). The endoplasmic reticulum was often associated with the areas of active wall synthesis (Fig. 46). Organelles passed from the mother spore into the germ tube (Fig. 47).

Dormant sporangiola generally are not vacuolate; however, the number and size of vacuoles increases during the germination of C. cucurbitarum (Fig. 38) and the number of lipid bodies is considerably less in germinating spores (Fig. 50). Paramural bodies were associated with the walls and plasmalemma (Figs. 49, 50). Patches of a light staining material, most likely glycogen (Fig. 37) and lomasomal figures were often observed in germinating spores (Fig. 41)

Discussion

The swelling of dormant spores prior to germination has been described by Marchant and White (1966) for Fusarium culmorum. Hawker et al. (1970) and Dykstra (1974) have made similar observations on species of Cunninghamella. Khan (1975) has reported that the spores of Cunninghamella echinulata do not swell prior to germination. In this study and in Poitra's (1955) the spores of C. cucurbitarum swell slightly prior to germination. According to Hawker and Abbott (1963), Marchant (1966b), Bracker (1966), Hawker et al. (1970) and Gull and Trinci (1971), the synthesis of new wall layers occurs at this time. The furrowed and coagulated appearance observed in C. cucurbitarum has been observed by Hess and Weber (1973) in Rhizopus arrhizus Fischer.

Hemmes and Hohl (1969), Grove and Bracker (1970), Bracker (1971), and Grove (1972), reported that the apices of actively growing hyphae and germ tubes were concentrated with cytoplasmic vesicles. They concluded that these structures were involved in hyphal extension. Bartnicki-Garcia (1973) suggested that vesicles contribute to the formation of new membranes and walls.

A new vegetative layer is formed over the emerging germ tube of C. cucurbitarum. Hawker et al. (1970) observed this same phenomenon and concluded that this area was a chemically changed part of the existing spore wall. Khan (1975) stated that this area was not chemically changed but new and different from any of the pre-existing walls. Other investigations have reported that the wall of the germ tube is continuous with a new layer which completely or partially surrounds the entire spore (Marchant, 1966a; Hemmes and Hohl, 1969; Hawker and Abbott, 1963; Hawker, 1966; Hawker et al., 1970; and Border and Trinci, 1970). Hawker and Hendy (1963), and Dykstra (1974) reported that in B. cinerea and C. vesiculosa the germ tube was an extension of the spore wall.

The present investigation shows that a newly formed vegetative layer becomes the wall of the germ tube and does not surround the sporangiospore. These observations corroborate the findings of Khan (1975) and support his supposition that this vegetative layer is new and not a part of any pre-existing wall layers.

Dykstra (1974), Hawker et al. (1970), and Bartnicki-Garcia (1968) have suggested that the mode of germination may play a significant role in determining taxonomic

relationships. Modes of germination have been shown to vary even among members of the same genera (Border and Trinci, 1970; Remsen et al., 1967; Tanaka, 1966; Stocks and Hess, 1970; and Heintz and Niederpruem, 1971). Perhaps many of the variations are artificial and can be resolved with improved fixation procedures. Until more conclusive evidence is available, the mode of germination cannot be used as a valid taxonomic character.

Chapter III

Figures 34-39. Swelling and initial germinating stages in Choanephora cucurbitarum.

Figure 34. Sporangiospores at several stages of germination. X500

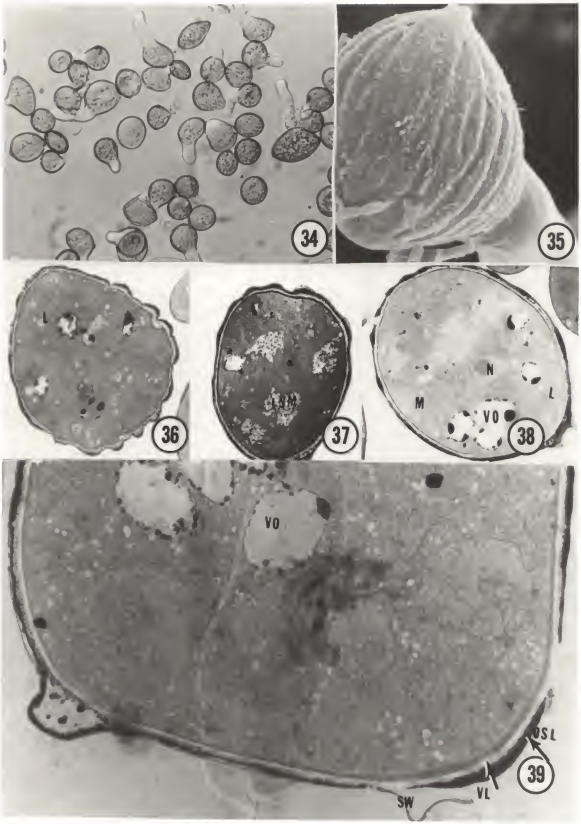
Figure 35. Germ tube emergence from lateral side of sporangiospore. X5,000

Figure 36. Sporangiospore with undulated walls. Lipid bodies (L). X4,600

Figure 37. Light staining material (LSM), spore in expanding phase. X4,600

Figure 38. Multinucleate spore (N). Mitochondria (M). Lipid (L). Vacuole (Vo). X4,600

Figure 39. Vegetative layer (VL) forming at germ tube apex. Large vacuoles (Vo). Outer spore layer (OSL). Spore wall (SW). X15,000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Chapter III

Figures 40-46. Stages in the development of the germ tube.

Figure 40. Vegetative layer (VL) thickens over apex. Outer spore layer (OSL). Inner spore layer (ISL). X66,000.

Figure 41. Lomasome (Lo) at germ wall (GW) apex. X75,000

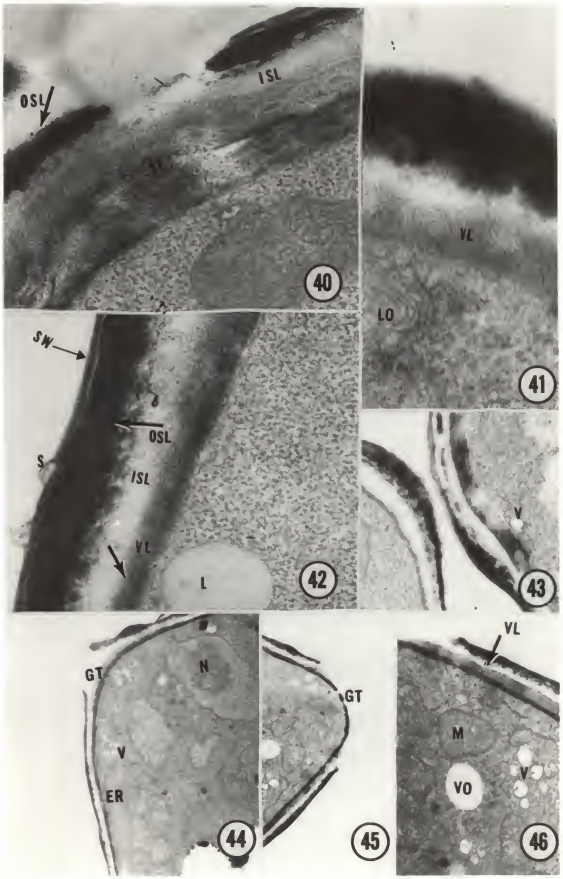
Figure 42. Vegetative layer ends (arrow). Spore wall (SW). Spines (S). Outer spore layer (OSL). Inner spore layer (ISL). Lipid (L). X66,000

Figure 43. Comparison between two unispored sporangiola germinating and during a quiescent stage.

Figure 44. Vesicles (V) and endoplasmic reticulum (ER) at germ tube (GT). Remnants of inner spore layer on germ tube. X12,000

Figure 45. Emerging germ tube (GT). X12,000

Figure 46. Vesicles (V). Mitochondria (M). Vacuoles (Vo). Vegetative layer (VL). X25,000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Chapter III

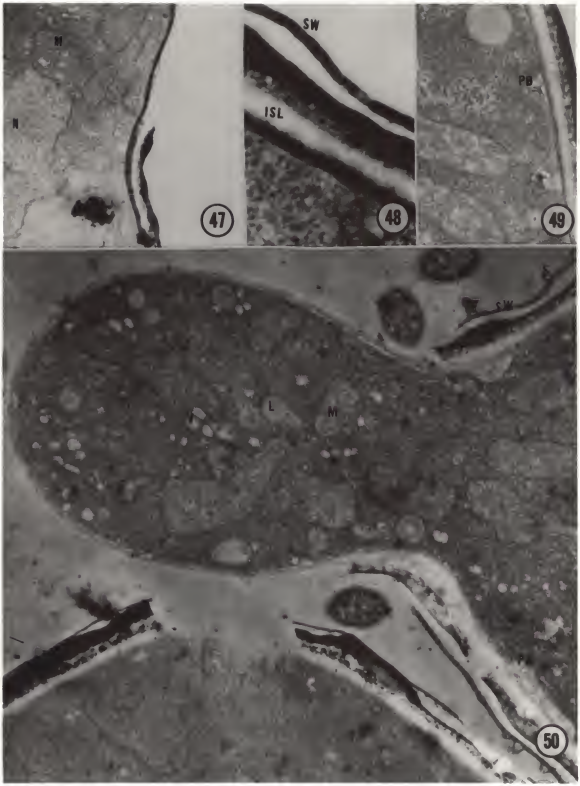
Figures 47-50. Stages in the germination of unispored sporangiolum. X20,000

Figure 47. Organelles move from mother spore into germ tube. X20,000

Figure 48. Sporangiolar wall (SW). Inner spore layer (ISL). X66,000

Figure 49. Paramural bodies (PB) associated with plasmalemma in sporangiospore. X10,000

Figure 50. Lipid bodies (L) in fully developed germ tube. Spines (S) on sporangiolar wall (SW). Vesicles (V) concentrated at apex. Mitochondria (M). X7,500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CHAPTER IV

MORPHOLOGY OF SPORANGIA AND SPORANGIOSPORES IN *Choanephora cucurbitarum*

Asexual reproduction in the Zygomycetes is by the production of various types of aerial sporangia. The acolumellate unispored sporangiola develop by a blastosporic process somewhat similar to many of the Deuteromycetes. The walls of the unispored sporangiola may be unfused, as in *Choanephora* (Poitras, 1955) or fused as in *Cunninghamella* (Khan and Talbot, 1975). The fused appearance of the spore wall and sporangiolar wall in *Cunninghamella* spp. has led to the term conidium in the Mucorales (Young, 1968; Hawker et al., 1970; and Dykstra, 1974). Khan and Talbot (1975) demonstrated that the spore wall and sporangiolar wall were separate. Certain species produce few-spored sporangiola, as in *Blakeslea* spp.(Thaxter, 1914). These reduced sporangia may or may not develop a columella, however, if a columella is formed it is usually rudimentary. The merosporangium is a cylindrical sporangium in which spores are cleaved out uniseriately and contains one to many spores (Benjamin, 1959). The merosporangium is characteristic of the

Kickxellaceae, unispored-bispored; Dimargaritaceae, unispored-bispored; Piptocephalidaceae, unispored-many spored and the multi-spored Syncephalastraceae. The merosporangial forms lack columellae but form various types of septa (Benny and Aldrich, 1972). The classic Mucoralean sporangium is globose to subglobose containing from one hundred to several thousand sporangiospores. The exterior may be smooth walled or roughened by deposits of calcium oxalate crystals.

The sporangiospores are formed by progressive cleavage (Schwarze, 1922; Bracker, 1966, 1968). Small vesicles coalesce to form numerous larger vesicles during cleavage. The larger vesicles form cleavage furrows in the cytoplasm. The multinucleate portions which are cleaved out are surrounded by a plasma membrane and the spore wall is secreted around the membrane and cytoplasm. The columella is formed prior to the completion of the spore forming process.

The sporangiospores have various colors, shapes, and ornamentations. Certain species produce hyaline tufts of appendages at both ends of the spore (Young, 1968; Bracker, 1968). The sporangiospore wall may be one to many layers as in Rhizopus spp. (Hawker and Abbott, 1963).

Multispored sporangia in the Choanephoraceae were discovered by Cunningham (1878). Although the sporangial form can be induced in culture, it has not been found associated with diseased tissue in the field.

Thaxter (1914) described Choanephora cucurbitarum but did not mention whether the sporangiospores were striate or smooth. Lefebvre and Weimer (1939) reported the striate nature of the sporangiospores of C. cucurbitarum.

Poitras (1955) stated that the striations on the sporangiospore wall of C. cucurbitarum had not been illustrated by Lefebvre and Weimer (1939). He proposed to illustrate these striations but did not. Young (1968) and Kawakami (1956) did not show the striate or appendaged condition of the sporangiospores of the Choanephoraceae they observed.

In view of the current lack of knowledge concerning the morphology of these spores and sporangia, the following study will present scanning and transmission electron microscopy of the propagules during dormancy and germination.

Materials and Methods

Light Microscopy

Diseased squash tissue was brought into the laboratory and isolates of Choanephora cucurbitarum were obtained after incubation of spore heads on squash agar (Chapter II). Hyphal tips were taken from these cultures and placed on one-half strength cornmeal agar. Plates were left at room temperature until sporangia developed.

Sporangia were taken at various stages of development, mounted in distilled water, lactophenol cotton blue or phloxine-KOH. Sporangiospores were obtained by applying pressure to the cover slip to force the sporangia open. Observations were made under phase and light microscopy.

Germinating sporangiospores were prepared according to the precedure reported by Dykstra (1974). Spores were removed from the suspension at the desired stages and placed on a slide and coverslipped. Germinating spores were viewed under light and phase microscopy.

Electron Microscopy

Sporangiophores with attached sporangia were placed on a thin layer of agar and covered with a second layer.

Agar blocks containing the specimens were cut, placed in buffered Karnovsky's (1965) fixative for 15 min at room temperature and one hour 45 min at 4 C. After washing in 1:1 v/v buffer (sodium cacodylate pH 7.0) and distilled water, blocks were post-fixed in 1% osmium tetroxide overnight at 4 C.

Specimens were removed from osmium and washed in water and buffer 1:1 v/v. The blocks were dehydrated through a graded alcohol series and acetone (100%), embedded in Spurr's (1969) embedding media, and polymerized in a 60 C oven for 24 hours.

Ultra-thin sections were cut on a Sorvall MT-2 ultramicrotome. Sections were floated onto 300 mesh, grids, post stained for 15 min in 2% uranyl acetate and 5 min in lead citrate. Observations were made on a Hitachi HU-11E electron microscope.

Scanning Microscopy

Sporangiospores with attached sporangia were placed on a millipore filter and fixed with buffered Karnovsky's (1965) fixative. The fixative was vacuumed off and replaced with a graded acetone series (25, 50, 75, 100, 100). Specimens were critical point dried and mounted on stubs with double-sided adhesive tape and coated with gold in a Hummer-II sputter coater.

Germinating sporangiospores were obtained by inoculating squash fruit and incubating at room temperature for 90 min. Infected tissue was fixed as above, critical point dried and gold coated in a Hummer-II sputter coater. All observations were made on a Zeiss scanning electron microscope.

Results

The multispored sporangia observed are similar to others observed in the Mucorales. The globose heads are held circinately on aerial sporangiophores (Figs. 51, 52, 55). Sporangia are light colored but turn black as the heads mature (Figs. 51, 52). The sporangial wall is spinulose (Figs. 55, 56, 64) and occasionally calcium oxalate crystals cover the surface (Fig. 54). A pyriform columella (Fig. 53) is formed at the apex of the sporangiospore and extends into the sporangium.

Sporangiospores are larger than unispored sporangiola of the same species and have bipolar hyaline appendages (Figs. 57, 60). The spore surface has longitudinal striations similar to the striations on monosporous sporangiola (Fig. 58).

The sporangiospore has two wall layers, an electron-dense outer layer and a lighter staining inner wall layer (Fig. 59). The furrows observed on the outer walls of monosporous sporangiola are not as prominent. The appendages resemble curled hairs in cross-section (Fig. 56) and in longitudinal-section (Fig. 60) they appear as two parallel electron-dense layers.

Sporangiospores are dispersed as the sporangium splits in half along a preformed suture (Figs. 55, 61, 62, 63). The spores are held together by a slimy substance (Fig. 64) and remain in mass upon complete dispersal (Fig. 65).

Germination is similar to that observed in monosporous sporangiola of the same species. The spores swell slightly (Fig. 66) and the germ tube emerges from the side of the spore (Figs. 66, 67, 68). The germinating spore is multinucleate, vacuolated, and contains numerous lipid bodies and mitochondria (Fig. 68). Endoplasmic reticulum and vesicles were often associated with the spore wall (Fig. 59).

Discussion

The sporangia and sporangiospores of Choanephora cucurbitarum (Berk and Rav.) Thaxter, vary in size. The

sporangial spines and bivalve sporangia are key taxonomic characters of the Choanephoraceae. Choanephora, Blakeslea and Gilbertella are the only species in the Mucorales which have longitudinally striate sporangiospores with bipolar appendages. Hesseltine (1960) and Cheng (1964) have suggested that Gilbertella is a link between the Mucoraceae and the Choanephoraceae because of its mucor-like zygospores.

Schwarze (1922) and Bracker (1968) have described the stages and cellular apparatus involved in sporangiospore formation. According to Bracker (1968), the appendages are formed during pre-cleavage, the columella during mid-cleavage and the mature spore is completely cleaved out by post-cleavage. Several stages described by Bracker have been observed by the author in C. cucurbitarum (unpublished).

Observations on the origin and number of wall layers in sporangiospores are not presented since the development of these walls was not observed. Observations have shown that the mature sporangiospore has two layers similar to the spore wall in monosporus sporangiola.

The appendages of Choanephora and Blakeslea were described by Bracker (1966) and Butler (unpublished). The

appendages, according to the authors, are attached to the outer spore wall. They are further described as ribbon-like, appearing as two parallel electron-dense layers in longitudinal-section and curled in cross-section. Similar observations on the appendages of C. cucurbitarum were made in this study.

Light microscopy, transmission and scanning electron microscopy reveal that these spores germinate by the lateral emergence of the germ tube and the germination rate is no different from monosporous sporangiola.

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Figures 51-55. Morphology of sporangia in Choanephora cucurbitarum.

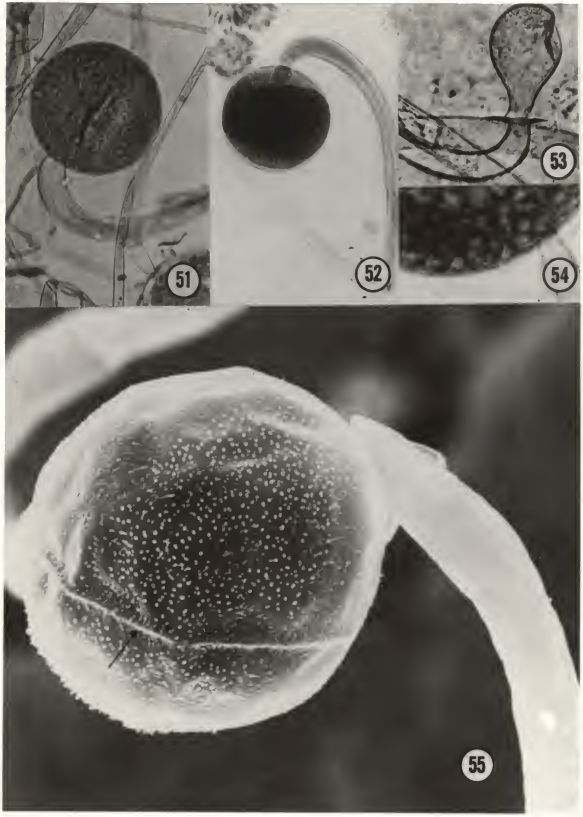
Figure 51. Cincinate sporangium--cream colored stage. X320

Figure 52. Sporangium turns black when mature. X270

Figure 53. Collared, pyriform columella. X150

Figure 54. Sporangia with sporangiospores. Calcium oxalate crystals on surface. X225

Figure 55. Bivalve nature of circinate sporangia. X1,400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Figures 56-59. Stages in the formation of sporangiospores.

Figure 56. Sporangiospores are cleaved out. Sporangial wall (SW) with spines (S) attached to surface. Appendages (A) in cross-section. X64,000

Figure 57. Bipolar appendaged sporangiospore. X750

Figure 58. Striate nature of sporangiospore. X2,500

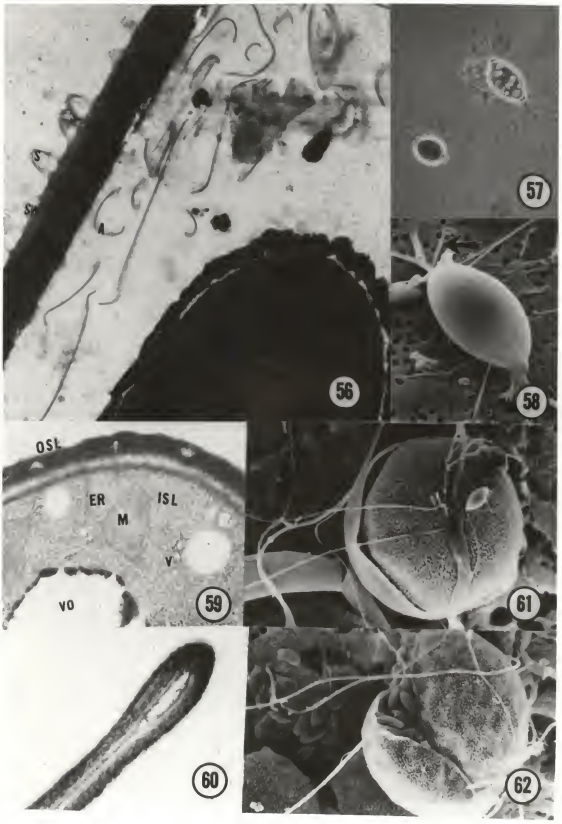
Figure 59. Endoplasmic recticulum (ER). Mitochondria (M). Vesicles (V). Vacuoles (Vo). Inner Spore layer (ISL). Outer spore layer (OSL). X25,000

Figure 60. Longitudinal section through an appendage.

Figures 61-62. Dehiscence of sporangiospores from sporangia.

Figure 61. Suture prior to dehiscence. X500

Figure 62. Sporangiospores escaping through suture. X500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Chapter IV

Figure 63-65. Dehiscence of sporangiospores from sporangia.

Figure 63. Suture widens as spores are released. X250

Figure 64. Note spinulose surface of sporangia. Sporangiospores held in slimy mass. X2,500

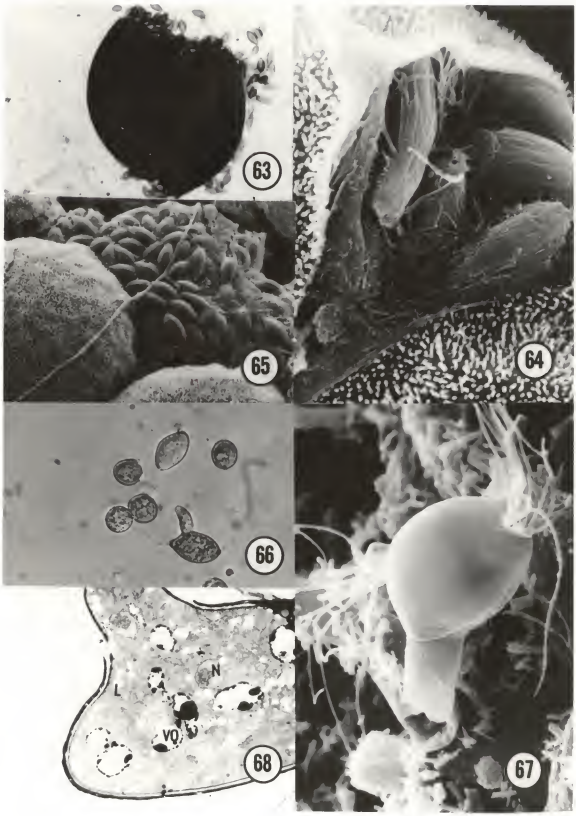
Figure 65. Sporangia completely split, spores at mouth of open suture. X500

Figures 66-68. Germination of sporangiospore.

Figure 66. Sporangiospores swell. Germ tube arises on lateral side of sporangiospore. X750

Figure 67. Bipolar appendages and striate sporangiospore.

Figure 68. Vacuoles (VO). Multinucleate Spore (N). Lipid bodies (L). X25,000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CHAPTER V

MORPHOLOGY AND DEVELOPMENT OF ZYGOSPORES IN *Choanephora cucurbitarum*

The sexual spores of the Zygomycotina, zygospores, form as a result of the fusion of two multinucleate progametangia and the subsequent fusion of their protoplasts. This process is called gametangial copulation (Alexopoulos, 1962).

Septa are formed between the progametangia and vegetative hyphae. The distal cell forms the young zygospore and the proximal cell becomes the suspensor cell. The resulting dark, thick-walled zygospore is enclosed in the zygosporangium. The suspensors are usually apposed or opposed and of equal (isogamous) or unequal size (anisogamous).

Blakeslee (1904) was the first to report on heterothallism in the fungi. He made this classic discovery while studying sexual reproduction in the Mucorineae. Since Blakeslee's report, zygospore cytology and germination has not been extensively investigated. The difficult task of inducing germination has probably contributed to this lack of scientific investigation.

Cutter's (1942a-b) investigations on the cytology of zygospores have contributed to much of our present knowledge. He concluded that four types of nuclear conditions occur in the Mucorales. The four types are listed as follows: (1) the Mucor pattern in which all functional zygospore nuclei undergo karycgamy followed by immediate reduction prior to the onset of dormancy; (2) the Rhizopus pattern wherein only a portion of the nuclei in the zygospore fuse and the other nuclei degenerate before the onset of dormancy, and reduction division is delayed until germination; (3) the Phycomyces pattern is characterized by nuclei associated in groups in the young zygospore and remaining associated until just prior to germination, when partial fusion of the nuclei occurs; and (4) the Sporodinia pattern in which nuclear fusion does not take place at any stage in the zygospore. Blakeslee (1906) reported on zygospore germination in members of the Mucorineae. Perhaps, Mosse's (1970a-c) studies on zygospore germination in the Endogonaceae are the most recent reports on zygospore germination. Couch (1925) credits Blakeslee with the discovery of heterothallism in Choanephora cucurbitarum. Weber and Wolf (1927) reported on heterothallism in B. trispora strains found in Gainesville, Florida.

Three members of the Choanephoraceae have been studied cytologically; Blakeslea trispora, Cutter (1942a), Choanephora cucurbitarum; Wolf (1917), Morie (1966), Blakeslea circinans, Morie (1966). Cutter (1942a) suggested that B. trispora adheres to the "Mucor" pattern but Morie (1966) observed that C. cucurbitarum and B. circinans follow the "Rhizopus" pattern of nuclear behavior described by Cutter (1942b).

Considerable attention has been given to the surface structure of zygospores in the Mucorales. Cunningham (1878) described the striate nature of the zygospores (epispore) in C. simsonii. Couch (1925) reported that the zygospores of C. conjuncta were striate and suggested that this character separated it from C. cucurbitarum. Poitras (1955) reported that Shanor had observed striations on the zygospore walls of C. cucurbitarum and concluded that it was synonymous to C. conjuncta. After observing zygospores of B. trispora, Poitras (1955) concluded that they too were finely striate.

Our knowledge of zygospore morphology and development has been increased with electron microscope studies of Hawker and Gooday (1968, 1969) and more recently by the transmission and scanning observations by Schipper et al. (1975),

O'Donnell and Ellis (1977), and Kirk (1977). In view of the recent advances in both transmission and scanning electron microscopy, a re-examination of the zygospores of C. cucurbitarum seems necessary. The following investigation will present light microscopy and scanning and transmission electron microscopic observations on the zygospores of C. cucurbitarum.

Materials and Methods

Light Microscopy

Many of the strains used in this study were isolated in Gainesville, Florida, in 1975. One of the mating types was isolated by the author from infected morning-glory used as scion on sweet potato root stock in Tuskegee, Alabama. This strain has temporarily been labeled (+) Atus while all other isolates have been labeled (-).

Spores or hyphal tips of each strain (+) and (-) were placed on opposite sides of a Petri dish. Plates were placed in the dark at room temperature for four days. Zygospores developed along a dark line where the two strains met.

Sections of agar containing zygospores were taken at various stages between the second and fourth day and

mounted in distilled water, lactophenol or phloxine-KOH for light and phase microscopy.

Electron Microscopy Procedures

Agar blocks containing zygospores were placed in buffered Karnovsky's fixative (1965) for 4 hours at 4 C. Blocks were washed and then post-fixed in 1% osmium tetroxide overnight at 4 C.

Blocks, after removal from osmium, were washed and dehydrated through a graded alcohol and acetone series. Specimens were embedded in Spurr's (1969) embedding medium and polymerized at 60 C overnight.

Thin sections were cut on a Sorvall-MT-2 ultramicrotome. Sections were placed on formvar coated grids, post stained in 2% uranyl acetate for 15 min and lead citrate for 5 min. Sections were observed on a Hitachi HU-11E electron microscope.

Scanning Electron Microscopy

Agar blocks containing zygospores were fixed in buffered Karnovsky's (1965) fixative for 4 hours at 4 C. After fixation zygospore blocks were washed in 1:1 v/v

buffer (sodium cacodylate pH7.0) distilled water, and dehydrated in a graded ethanol and acetone series. Specimens were critical point dried and gold coated in a Hummer-II sputter coater. Blocks with zygospores were placed in a Zeiss scanning electron microscope for observations.

Results

In paired cultures, hyphae of opposite mating types become wrapped and entangled at the zone of merger (Fig. 68) and bulges or swollen areas are visible.

Progametangia differentiate on aerial hyphae termed zygophores. Zygospore initiation occurs as two nearly isogamous progametangia contact at their apices (Fig. 69). A septum is formed distally from the contact point (Figs. 69, 71, 73). The proximal cell forms the gametangium and the remaining part forms the suspensor (Figs. 70, 72). At the point of contact the walls between the two gametangia dissolve centripetally (Fig. 70). Gametangial copulation resulted in the complete fusion of the protoplast (Figs. 71, 72).

The gametangium enlarges (Fig. 72) and secondary thickening of the septal wall begins after apical fusion

(Figs. 74, 75). The septal plate is transversed by perforation (Fig. 74) similar to plasmodesmata in higher plants. The plasmalemma is undulated in the gametangium and suspensor (Figs. 70, 72). Large amounts of endoplasmic reticulum, vesicles and microbodies can be seen in the septal area (Figs. 70, 73).

The zygospore enlarges to become spherical on apposed, tong-like suspensors (Fig. 76). The smooth wall surface begins to slough off due to pressure from within (Fig. 77). Under light microscopy the zygospore can be seen within the zygosporangial wall (Fig. 78). The outer zygosporangial wall sheds or cracks to reveal the striate nature of the zygospore (Fig. 79).

In ultra-thin sections the zygosporangial wall is smooth, hyaline and thin. Vesicles, lomasomes and a light staining inclusion are associated with the zygospore wall (Fig. 80). The zygospore wall appears thick and dark-walled. A multivesicular body can be seen near the zygospore wall (Fig. 81).

Discussion

Hawker and Gooday (1967) have described in detail the delimitation and formation of septa in Rhizopus sexualis (Smith) Callen. The fusion vesicles have been described. Fusion vesicles described by Hawker and Gooday (1967), Hawker and Beckett (1971), O'Donnell et al. (1977), were not as prominent in the septal region of C. cucurbitarum. Failure to observe these vesicles can be attributed to the developmental stage under observation. Vesicles in the septal area of C. cucurbitarum are comparable to those in hyphal tips (Grove and Bracker, 1970) and germ tube apices (Bracker, 1971). In the species they observed, new walls and membranes were contributed by these vesicles.

Secondary thickening of the septal wall in C. cucurbitarum appears to be greater on the suspensor side of the septum. O'Donnell et al. (1976) observed similar thickening in Phycomyces blakesleeanus Burgeff. However, Hawker and Gooday (1967) and O'Donnell et al (1977) reported secondary thickening in the septal area of Rhizopus sexualis and Gilbertella persicaria to be asynchronous and greater on the zygospore side of the septum.

Plasmodesmata similar to those in higher plants are formed through the septal plate in some mucoraceous fungi

(Hawker and Gooday, 1966; Hawker and Gooday, 1967; Hawker and Beckett, 1971; O'Donnell et al., 1976; and O'Donnell et al., 1977). Powell (1974) and Robards (1971) suggested that these pores are formed as endoplasmic reticulum cisternae become trapped in the developing septa in chytrids and higher plants, respectively. It is believed that septa provide continuity and communication between adjacent cells. Hawker and Gooday (1967) reported that the septa formed before the fusion walls dissolved and that fusion is centripetal. According to the authors dissolved wall material from the fusion zone may be transported to the forming septum. The septal plate in C. cucurbitarum is similar to the multiperforate septa in other Mucorales.

O'Donnell et al. (1977) stated that the warty layers of G. persicaria are formed by the deposition of secondary wall material within the inner primary zygospore wall and this secondary wall material is similar to the secondary thickening in the septum. Hawker and Beckett (1971) reported similar observations on the formation of warts in R. sexualis. The ornamented layer in Phycomyces (O'Donnell et al., 1976) differs from the above in that the ornamented layers are formed within the secondary zygospore wall.

According to the above authors the gametangial wall sloughs away to expose the various ornamentations, and fragments of the gametangial wall which often adhere to the newly formed ornaments. O'Donnell et al. (1977) have summarized the common features of zygosporogenesis and they are listed as follows: (1) warts are initiated in the equatorial region of the prozygospore adjacent to the disintegrating fusion septum, and (2) endoplasmic reticulum and Golgi cisternae are associated with wart formation. Remnants of the outer primary wall adhere to the warty layers but the inner primary wall cannot be discerned in mature zygospores.

Benjamin (1959) has described the typical mucoraceous zygosporangium as roughened, irregularly thickened and highly pigmented, usually brown, orange or black. The zygosporangium is surrounded by a smooth, thick-walled zygospore. He also reported that the zygospores of Choanephora, Mortierella, Radiomyces and all species of Dimargaritaceae, Kickxellaceae, and Endogonaceae are relatively thin-walled, smooth and nearly hyaline. The endospore or zygospore is described as striate, thick-walled and dark colored.

The results of light microscopy, scanning and transmission electron microscopy in the present investigation concur with the light microscopy observations of Benjamin

(1959), scanning electron microscopy by Kirk (1977), and many of the transmission electron microscopy observations made on other Mucoralean species (Hawker and Gooday, 1967; O'Donnell et al., 1976; O'Donnell et al., 1977).

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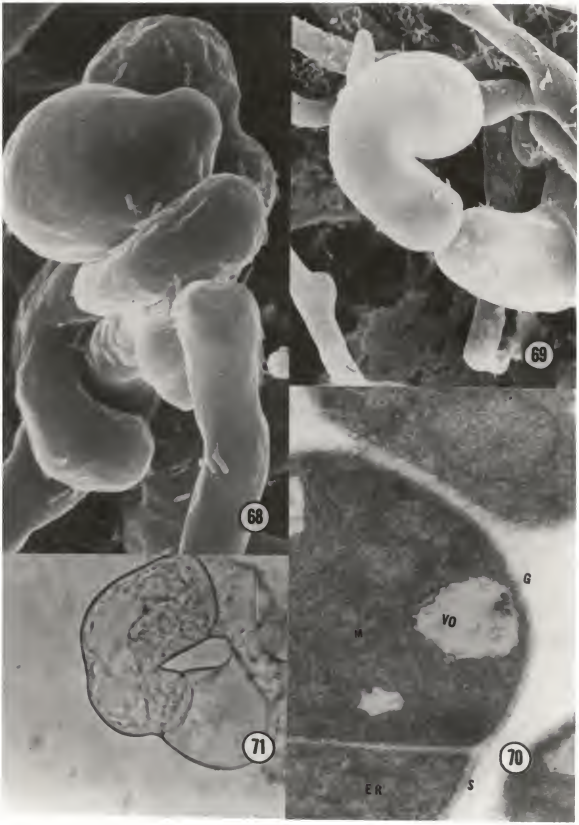
Figures 68-71. Stages in the formation of the zygospore.

Figure 68. Entangled hyphae of opposite mating types. X6,000

Figure 69. Apical contact of progametangia. X8,000

Figure 70. Early stage of septum formation. Dissolution of apical walls. Suspensor (S) Gametangium (G). Vacuole (Vo). Endoplasmic reticulum (ER). Mitochondria (M). X23,000

Figure 71. Fusion of protoplast after dissolution. X1,000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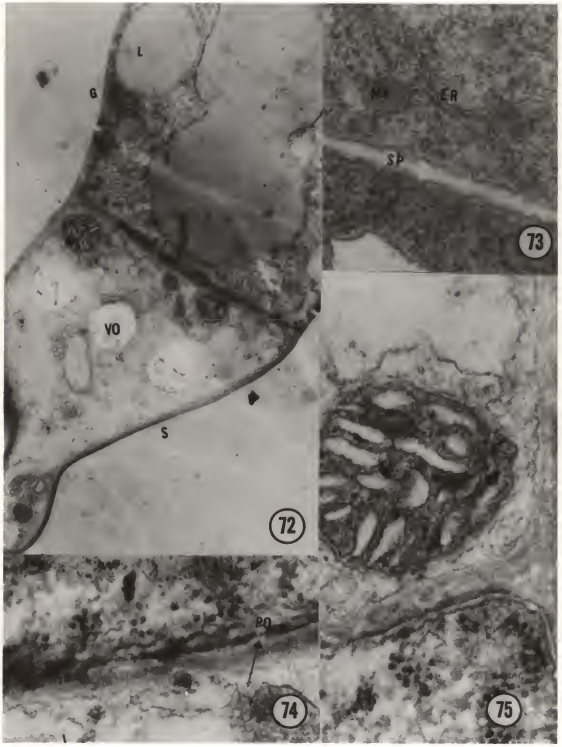
Chapter V

Figures 72-75. Stage in the formation of the zygospore.

Figure 72. Mitochondria (M). Lipid body (L). Vacuole (Vo). Suspensor (S). Gametangium (G). X18,000

Figure 73. Septal plate (SP) before perforation (note the undulation). Endoplasmic recticulum (ER). Microbody (MB). X60,000

Figure 75. Secondary wall thickening along lateral side. X96,000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Chapter V

Figures 76-81. Stages in the formation of zygospore

Figure 76. Zygospore enlarges on tong-like suspensor. X8,000

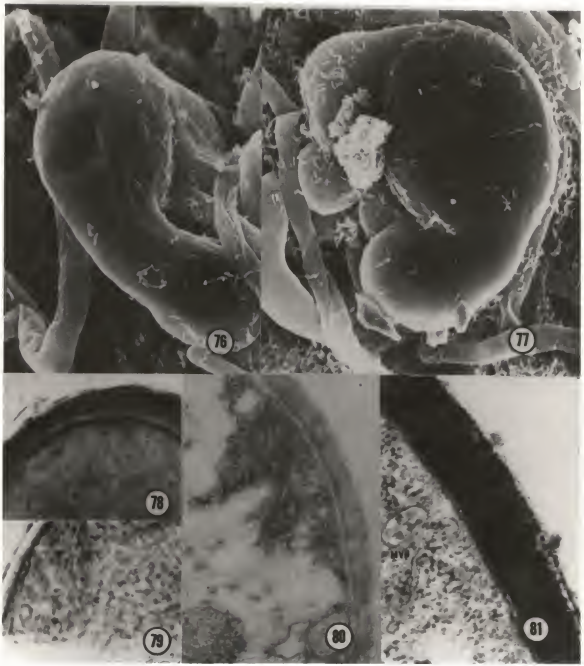
Figure 77. Mature zygospores. X6,000

Figure 78. Zygosporangium and zygospore. X1,000

Figure 79. Striate zygospore surface. X1,000

Figure 80. Smooth-walled zygosporangium . X75,000

Figure 81. Mature dark-walled zygospore Multivesicular body (MVB). X75,000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CHAPTER VI

SURVIVAL OF *Choanephora cucurbitarum* AND ITS RELATIONSHIPS WITH ITS HOSTS, *Cucurbita* spp.

Cunningham (1878) was the first to discuss the parasitic nature of fungi in the genus *Choanephora*. Today *Choanephora* spp. are recognized as pathogens of many species of flowering plants. Couch (1925), Wolf (1917), Lefebvre and Weimer (1939), Toler and Dukes (1965), and Fery and Cuthbert (1972) have reported pathogenic species of *Choanephora* in the United States. Such species were reported earlier in India by Palm and Jochems (1924), Tai (1934), and Sinha (1940); in South America by Moeller (1901) and Dantas (1953); and in South Africa by Deighton (1936).

The *Choanephora*-induced blossom-blight disease of cucurbits may be relatively unfamiliar to plant pathologists not residing in tropical to subtropical regions in which the disease is endemic. There is also a paucity of literature on blossom blight of cucurbits. Poitras' (1955) survey of over forty papers on the genus *Choanephora* revealed that only twenty-two dealt with the pathology of

of the disease its members cause. Only eleven papers on the pathology of Choanephora have appeared since 1955, and most of them are only brief citations of disease occurrence.

In this chapter, I treat two subjects: the survival of Choanephora cucurbitarum between cropping seasons and the pathogenicity of that fungus to squash.

Survival of Choanephora cucurbitarum Between Growing Seasons

Published accounts of how Choanephora cucurbitarum survives between growing seasons have not been found. Of its four spore forms, chlamydospores seem the most likely organs of survival, but the monosporous sporangiola--particularly in tropical and subtropical regions--might persist from season to season. Further, the fungus might survive as a saprophyte in decaying crop-plant residues in the soil. Efforts were made to isolate C. cucurbitarum from field soils in which squash had been grown and from another soil in which squash was already in blossom. Tests were made on the day of sampling, or the samples were stored in the refrigerator at 4 C and tested within four days.

Three procedures were followed: the soil-plate technique of Warcup (1950), a baiting method and the direct-plating technique. Soil plates were prepared by placing 5-15 micrograms of fresh field soil in each of fifteen sterilized Petri dishes. Approximately 10 ml of melted 2% water agar, cooled to about 45 C, was added to each plate, which was swirled to spread the soil samples. Plates were kept in the laboratory at 25 ± 3 C, and were observed daily for three days. The soil-plate technique was unsatisfactory for the isolation of C. cucurbitarum from the soil. Fusarium spp. and other Deuteromycetes quickly colonized the plates; Choanephora was never found and other members of the Mucorales were seldom observed.

Two baiting techniques were used. In the first small pieces of squash flower or fruit were surface sterilized and placed in Petri dishes filled with soil. Plates were kept in the laboratory at 25 ± 3 C. As with the soil-plate method, Choanephora could not be isolated because the bait tissues were quickly colonized by saprophytic Deuteromycetes and mucors. In the second baiting technique, soil samples weighing 5-15 micrograms each were placed on sliced squash fruit in sterile Petri dishes. Plates were kept in the laboratory at 25 ± 3 C. C. cucurbitarum was

isolated from all fruit slices contaminated with soil from the field where squash had grown; the parasite was also found in fruit slices contaminated with soil from the field where squash was in blossom. These results prove that C. cucurbitarum survives in the soil between cropping seasons.

In the direct-plating method, soil samples were moistened with distilled water and 5-15 microgram samples were placed separately in Petri dishes containing one of the following culture media: potato-dextrose agar (PDA), phytone-yeast-extract agar, Emerson's yeast agar (YPS), or squash decoction agar. Plates were left overnight at 25 $\pm$ 3 C. Plant debris,washed to remove soil, were also placed on each of the culture media. Hyphae or sporulating heads present after 24 hours, were subcultured for further study.

C. cucurbitarum grew from all infested soil samples and from all tissue samples on phytone-yeast-extract agar. This culture medium proved to be highly selective for the initial isolation of C. cucurbitarum. Although the fungus grew well in pure subcultures on the other media used, it could seldom be isolated in pure culture from those media.

Although the form or forms of survival structures were not viewed, results of the tests just described show that C. cucurbitarum survives on or within the soil and in plant debris. Survival in alternative hosts is unlikely since most of these are annual species. The use of phytone-yeast-extract agar as an isolation medium should be useful in further research on the ecology or survival of C. cucurbitarum in the soil.

Pathogenicity of C. cucurbitarum to Squash

Symptoms of Blossom Blight and Fruit Rot of Squash

The most destructive and common symptoms of Choanephora cucurbitarum on squash are blossom blight and fruit rot. Infection of the blossoms is apparent the morning after opening of the blossoms. They appear discolored, water-soaked and transparent. Within 48 hours of the onset of infection, dense, white sporulating heads of the fungus can be observed. These heads quickly turn dark brown or black, and mycelia can then be seen.

Wolf (1917) states that when staminate flowers are attacked, the pathogen passes from the corolla and sporulates along the pedicels; in pistillate flowers,

however, the pathogen passes from the corollas into the young fruit. Infected male flowers are blighted and fall away, but female flowers,although infected, may remain attached to the diseased fruit. After invading the fruit, the fungal mycelia ramify throughout the fruit tissues, converting them into a water-soaked mass. Ultimately, rot and leak follow hydrosis, and signs of the pathogen appear. The entire fruit may become covered with a dense mat of cottony mycelia and dark spores.

Review of the Literature

Because different spores of Choanephora cucurbitarum may be important in its parasitism, it seems appropriate to review the facts on that subject. The most familiar spore form in the choanephoraceae are the monosporous sporangiola (Wolf, 1917) often referred to as "conidia." The sporangiolophores are erect and terminate in a dilated primary vesicle. Several branchlets radiate from the primary vesicle and form secondary vesicles (ampullae) at their distal ends. Monosporous sporangiola are produced by a blastosporic process on the surface of these secondary vesicles. The spores are oval to elliptical, with longitudinal striations over their surfaces. The

sizes of the spores may vary from 8-10 microns x 15-25 microns. A small pedicel is formed at the point of detachment of spores from ampullae.

Large circinate sporangia, growing among the other spore forms but on separate hyphae, can often be observed in culture. Sporangia are white when young but quickly turn black with age. The sporangial heads are columellate and are held at the apex of slender spoangiophores. Dehiscence of spores occurs when the sporangia splits along a preformed suture. The bipolar appendaged sporangiospores are dispersed in a slimy mass. They are ovoid to elongate with longitudinal striations over their surfaces. This spore form is larger than monosporous sporangiola, measuring 10-15 microns x 18-30 microns. Chlamydospores are dark elongate structures that form on hyphae in older cultures. Cunningham (1878) reported that these structures germinate to produce multispored sporangia in C. infundilifera. Wolf (1917) stated that he did not observe germination of this spore form in C. cucurbitarum. Other observations on this asexual spore form have not presented additional data.

Zygospores form on undifferentiated hyphae between two apposed, tong-like suspensors. According to Benjamin

(1959), the zygosporangium is thin-walled and hyaline and surrounds a dark, thick-walled, striate zygospore. Zygospores are usually 50-90 microns in diameter.

Next, I consider briefly the subject of fungitropism, for this could play a part in the parasitism of squash flowers by Choanephora cucurbitarum. Carlile (1966) has described the spatial orientation of micro-organisms as tropisms when an agent influences the direction of their growth. The agent may be chemical or physical. Bandoni (1965) and Werkman (1974) have reported on the significance of oriented responses in the sexual reproduction of certain fungi. Royle and Hickman (1964), and Zentmeyer (1961) have reported positive chemotropism for plant roots by Pythium aphanidermatum Rands and Phytophthora cinnamomi (Edson) Fitzpatrick. Standler (1952, 1953) reported a negative autotropism in Rhizopus nigricans Ehrenberg. According to Standler (1952), Fulton (1906), and Graves (1916), the positive chemotropism observed by many investigators was in fact, autotropism. Autotropism is the response of germ tubes toward each other and negative autotropism is the growing of germ tubes away from each other into uncolonized media. Jaffe's (1966) studies on Botrytis cinerea Persoon ex Fries indicated that carbon

dioxide at a concentration of 0.3-3% caused negative autotropism. Carlile (1966) concluded that reports by Jaffe (1966) and Standler (1962) on the "clumping effect" suggested that high densities of spores cause negative autotropism and low spore density causes positive autotropism due to increased carbon dioxide produced under high spore density.

Parasitism by species of Choanephora induces necrotic symptoms, and the fungus grows both intercellularly and intracellularly; there was no evidence of cellulose secretion by the fungus (Wolf, 1917). Later, however, Gupta (1967) showed the production of cellulolytic enzymes by C. cucurbitarum, and suggested that this production was constitutive. Chahal and Grover (1972) reported that isolates C. cucurbitarum produced pectin-methylesterase (PME) and pectin depolymerase (DP), but did not produce polygalacturonase (PG). They concluded that PME and DP were involved in tissue maceration during pathogenesis; they also stated that virulence in their isolates was correlated with pectolytic enzyme activity.

Palm and Jochems (1924) reported that wounding facilitated infection of Amaranthus leaves, but that penetration could easily take place through the uninjured abaxial leaves.

Palm and Jochems (1924) and Sinha (1940) reported observing unispored sporangiola and multispored-sporangia in the field. The only asexual spore form of Choanephora cucurbitarum ever observed by me in the field is the unispored sporangiolum ("conidium"). It differs from the multispored forms only in morphology and spore development. According to Ingold (1970), unispored sporangiola are usually wind-dispersed. He stated that spores formed in a slimy "spore-drop" similar to that in multispored sporangia of Choanephora spp. are usually dispersed by insects. Frank and Slater (1956) reported that 98% of the insects visiting Cucurbita spp. were Hymenoptera or Coleoptera, and only 2% of the Hymenoptera were honey bees. According to these authors, the most important Hymenoptera were Xenoglossa strenua and Peponapsis priunosa. These species are commonly referred to as squash bees. They have evolved with Cucurbita and obtain their pollen solely from indigenous and domestic Cucurbita species.

Cunningham (1878) described the atmospheric conditions necessary for the development and spread of disease incited by C. cucurbitarum. He stated that disease development was greater during periods of high moisture in the air. He stated that "clear weather, cloudless nights and drenching

dews furnish the circumstances under which it [the disease] commonly occurs. Constant heavy rain or absence of dew are apparently equally repressive to its development." Later reports by Thaxter (1917), Wolf (1917), Palm and Jochems (1924) and Lefebvre and Weimer (1939) supported the conclusions drawn by Cunningham (1878).

Weber (1932) suggested that many diseases of pepper, including blossom blight, could be controlled, at least in part, by protective spraying with copper fungicides. Chahal and Grover (1972) investigated the efficiency of eight fungicides, *in vivo* and *in vitro* against *Choanephora cucurbitarum*. They concluded that all of the fungicides tested were better protectants than eradicants, but that Dithane Z-78 (Zineb), Thiram, and Cumen (Ziram) were also effective as eradicants. Dithane M-45, Dithane Z-78, Cumen, Bisdithane, and Thiram controlled the disease best when applied before inoculation. Weekly applications of Zineb are currently recommended in Florida to control blossom blight and fruit rot of cucurbits.

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Experiments on Aspects of the Pathogenic Life Cycle of Choanephora cucurbitarum

The purpose of this research was to investigate several aspects of blossom blight of cucurbits in Florida to determine: (a) which spore forms are involved in the dissemination of the pathogen, (b) the agent(s) of secondary spread, (c) whether varietal resistance exists, (d) the site and mode of entry, and (e) whether or not the site of entry is related to a chemotropic response of the germ tube to the host tissues involved.

Pathogenicity of Choanephora cucurbitarum to horticultural varieties of squash

Half-acre plots of four varieties of squash (Cucurbita pepo L.) grown by L. H. Halsey on the Horticultural Farm of the University of Florida were observed during 1977 for blossom blight. The summer varieties "Scallopini" and "Patty-Pan" were followed by two autumn varieties, "Dixie" and "Sundance." All varieties were examined twice weekly, beginning with the onset of flowering and ending at the time of the harvest of fruit. All four varieties were susceptible, the disease being particularly severe in the male flowers of the summer varieties (Fig. 84). Infections of female flowers (Fig. 85) were usually restricted to floral parts.

Pathogenicity of *Choanephora cucurbitarum* to excised flowers of squash

Flowers from squash plants grown in the field or in a greenhouse were excised and treated for 1 min in 10% Clorox. Flowers were rinsed and placed in sterile 250 ml flasks each of which contained enough sterile distilled water to immerse the pedicels. The flowers were inoculated on both surfaces of the petals and around the floral tubes with 5-15 micrograms of *Choanephora*-infested soil. Six replicates of the male and female flowers were covered with plastic to maintain 100% humidity and were left at 25 $\pm$ 3 C. Small sections of petal tissues were mounted on clean microscope slides, inoculated with spores and incubated up to 3 hours in Petri dishes lined with wet filter paper.

Male and female flowers inoculated with *Choanephora*-infested soil became diseased within 72 hours. Male flowers usually abscissed because of intense hydrosis and rotting when soil was inoculated around the floral tubes or on the exterior and interior surfaces of the corolla. Approximately 24 hours after soil inoculations around the floral tube, symptoms characteristic of blossom blight resembled those observed in the field. However, symptoms appeared

later when petals were inoculated. Flowers of uninoculated control plants withered and died but never showed symptoms of blossom blight and fruit rot. Microscopic observations revealed that the pathogen can penetrate directly into the adaxial surface of petals.

Chemotropic effects of flower tissues of squash on Choanephora cucurbitarum

The affinity of C. cucurbitarum for the floral tissues of squash suggested the possibility that such tissues exert a positive chemotropic effect on the parasite. Two lines of experimentation were followed in an effort to test this possibility. In some experiments, small pieces of vegetative and floral organs and pollen grains from species of Cucurbita were placed in Petri dishes containing 2% water agar. Sterile distilled water was poured into a 7-day-old culture of C. cucurbitarum on Emerson's YpSs (YPS) and the surface was scraped with a sterile glass rod to release the spores. Spores drawn into a sterile Pasteur pipette were placed randomly around the plant tissues and pollen grains. Plates were observed every 10 min for 90 min with a light microscope. In other experiments, small , thin sections of vegetative and floral organs of Cucurbita spp.

were placed on clean microscope slides and were inoculated with a suspension of spores from a 7-day-old culture of C. cucurbitarum. Slides were placed in Petri dishes lined with wet filter paper to increase the humidity during incubation; they were observed with the light microscope after 90 min.

Germ tubes from spores of C. cucurbitarum seemed chemotropically attracted to some floral tissues of squash and repelled by exudates from other tissues. Female tissues such as corolla (Fig. 98), fruits (Fig. 99), and styles (Fig. 97) induced positive chemotropic responses to germinating spores. In the male flowers, the stamens induced (Figs. 94, 95) a positive response, whereas the male corolla exudates induced negative chemotropic responses (Fig. 96). Spores inoculated around leaf tissue did not germinate within 90 min.

The results from the random inoculation of spores around squash tissue showed that spores germinated on all floral tissues after 90 min. Fewer spores germinated around leaf tissue and germtubes did not emerge until approximately 4 to 5 hours after incubation. Spores inoculated onto 2% water agar failed to germinate. Squash pollen grains induced spore germination similar to that

obtained by male and female floral tissues when they were added to plates containing 2% water agar or leaf decoctions. These results indicate that certain flower tissues of squash exert positive chemotropic effects on the germ tubes of Choanephora cucurbitarum. Moreover, pollen grains and corollas stimulate spore germination.

Growth of C. cucurbitarum on the vegetative and floral extracts of squash

Because floral tissues stimulated spore germination and were chemotropic to germ tubes of C. cucurbitarum, it seemed possible that extracts from flower tissues would stimulate vegetative growth of the fungus.

Liquid extracts and agar decoctions of floral parts and leaf tissue were prepared by adding 25g of tissue to 500 ml distilled water; the mixture was boiled for one hour. After boiling, the solution was filtered through four layers of cheesecloth and made up to 500 ml again with distilled water. One hundred milliliters of each extract were placed in each of these 250-ml flask. Four grams of agar were added to the final 200 ml of each extract. Flasks containing liquid extracts and agar decoctions were autoclaved for 15 min, 20 p.s.i. and 121 C.

Inoculum was prepared by pouring distilled water over cultures of C. cucurbitarum. Spore suspensions withdrawn into a Pasteur pipette were added to cooled liquid extracts and to solidified decoction plates. Agar decoction plates were incubated at 25 ± 3 C. Liquid extracts were incubated for 7 days at 25 C. After this time the mycelium from each flask was filtered, dried overnight at 60 C, and weighed.

Mycelial growth in liquid extract from various floral parts was variable. Greater growth was found on extracts of floral parts than on those of leaves (Table 1). There was little growth on extracts of the bud stage of female flowers, but mycelial growth on extracts of stamens and those of complete male flowers was greater than on those of the other male tissues.

Sporulation after 24 hours was greater on decoction agar of all female tissues (Figs. 89, 91, 92), as compared to those from male tissues, leaves, and controls. Decoctions of male corollas (Fig. 90), complete flowers, and male buds supported sparser growth than that from stamens (Fig. 88) which were comparable to the growth supported by decoctions from female tissues. Leaf extracts and controls produced mycelia that failed to sporulate (Fig. 93).

Table 1

Relative Amounts of Growth, Sporulation and Mating Vigor of Zygospores Cultured on Media Containing Flowers and Leaves of Squash Plants

Decoctions of	Milligrams of Mycelium after Seven Days*	Sporulation after 24 Hours**	Zygospore Mating Vigor on Decoctions (1 Week)***
Male Corolla	10	+	3
Female Corolla	17	++	5
Male Flower	16	+	7
Female Flower	18	++	8
Pistils	18	++	9
Stamens	22	++	10
Male Buds	11	+	2
Female Buds	8	++	4
Fruit	16	++	6
Leaves	7	--	1
Control	3	--	0

*Growth of mycelium in seven days at 25 C, in 100 ml extract in 250 ml flask.
**(++) = strongest, (+) = strong, (-) = no sporulation.
***--Number of zygospores per cm^2:

1 = 100	4 = 900	7 = 1400	10 = 2000
2 = 200-300	5 = 1000	8 = 1600	
3 = 700	6 = 1200	9 = 1700	

Zygospore mating vigor on the various floral extracts almost paralleled the mycelial growth obtained from liquid extracts. In both experiments, extracts from female buds, male corollas, and male buds ranked seventh, eighth, and ninth in the production of mycelium and zygospore mating vigor. Extracts from stamens, pistils, female flowers, and fruit tissues in each of the two experiments showed comparative results. Of the male tissues observed, extracts from male corollas and male buds showed the least stimulation to zygospore production. Leaf decoctions and control plates failed to produce zygospores in both experiments.

Isolation of C. cucurbitarum from bees

Results of experiments reported earlier in this chapter incriminate the soil and plant debris as sources of inoculum for the primary cycles of pathogenesis of C. cucurbitarum. As for secondary cycles, the observation of unispored sporangiola suggests that wind is the principal agent of spore dispersal. The association of beetles and pollinating insects with squash, however, raises the question of whether bees and other insects might also be important as agents of dispersal. Accordingly, bees were examined to learn whether they were contaminated by spores of the parasite.

Bees and beetles were collected by mechanically closing the ends of blossoms after the insects had entered the lower area of the floral tubes. The blossom and bee were placed in Zip-lock plastic bags and brought back to the laboratory. On the following day, bees and beetles could be removed from each blossom and placed separately in a sterile test test tube containing one milliliter of sterile distilled water. The tubes were shaken to facilitate the release of spores. After shaking, two 0.5 ml samples were placed on phytone-yeast-extract agar and were incubated at 25 $\pm$ 3 C. Hyphal tips from these plates were transferred to half-strength cornmeal agar (CM) or squash decoction agar for further observations. Bees were also placed directly onto the media and incubated at 25 $\pm$ 3 C.

Isolation by washing was effective in isolating Choanephora spp. from nine of the twelve bees used in this experiment. Two isolates sporulated within 24 hours and hyphal tips were taken from the seven non-sporulating cultures. Hyphal tips were subcultured onto one-half strength cornmeal agar or squash agar to induce sporulation. Light microscopy observations on drops of the suspension revealed that the only spore forms associated with bees and beetles were monosporous sporangiola and these were often

in a germinated state. Eight out of the twelve blossoms visited by the same bees showed signs and symptoms of Choanephora spp. infection within 72 hours when incubated in the laboratory at room temperature.

Isolation of Choanephora could be made with ease by placing bees directly on phytone-yeast-extract agar. Sporulation occurred on phytone-yeast-extract agar within 24 to 48 hours. Twelve of the sixteen blossoms under observation became diseased within 72 hours.

Conclusions

The following conclusions can be drawn concerning the pathogenesis of C. cucurbitarum to squash in northern Florida: (a) monosporous sporangiola were the only spore forms associated with disease in the field, (b) germination and infection into squash may be related to a chemotropic response of C. cucurbitarum germ tubes toward certain floral tissues, (c) the pathogen can penetrate directly into petal tissues, (d) the degree of sporulation on various leaf and floral tissues varied, and (e) bees could be involved in the spread of disease in the field.

Discussion

Results from the present study suggest that, in Florida, Choanephora cucurbitarum can survive from one crop season to the next as a saprophyte in soil and host debris. Plant pathogens often over-winter or survive as specialized resting structure such as zygospores, but such spores were not encountered on diseased squash tissue in Gainesville, Florida. Cunningham (1878) and Wolf (1917) are the only authors to report having observed this spore form in the field. All other observations on these structures have been in culture. Based on these findings, it is suggested that zygospores play no role or only a minor role in the disease as it occurs in northern Florida. Survival in alternative perennial hosts is unlikely, since there are only six indigenous species of wild Cucurbitaceae in northern Florida, and they appear to be annuals (Ward,[1] personal communication). Poitras (1955) published a lengthy list of all known hosts of C. cucurbitarum and none of the six genera of wild Cucurbitaceae of northern Florida was cited.

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[1]Prof. Daniel Ward, Curator of Spermatophytes, Department of Botany, University of Florida, Gainesville.

These studies indicate that the primary cycle of C. cucurbitarum in Florida is initiated primarily by the contamination of squash flowers with Choanephora-infested soil particles or particles of decaying host tissues. Splashing rain or other forms of free water might disperse the pathogen in the field.

Mansfield and Deverall (1971) concluded that the ability of B. cinerea spores to overcome the inhibitory action of wyerone acid, a fungitoxic compound in infected broad beans, is due to the presence of pollen. They suggested that pollen may play a role in stimulating infections of other plants. A similar mechanism may exist in C. cucurbitarum, since the addition of pollen stimulated germination of unispored sporangiola on water agar and squash vegetative tissue.

Carlile (1966) has summarized the biological significance of oriented responses. He suggested that the random despersal of spores may be compensated for by their environment. He stated that hyphal anastomosis observed by Buller (1933) and the development of hyphae within a colony (Butler, 1961) may be examples of positive and negative autotropism, respectively. The active agent responsible for positive chemotropism has not been identified, however,

positive chemotropism in pollen grains has been attributed to increased wall plasticity (Rosen, 1961) and negative chemotropism to decreased plasticity (Standler, 1952).

Zentmeyer (1961) and Royle and Hickman (1964) have shown that the germ tubes of Phytophthora cinnamomi and Pythium aphanidermatum, respectively, are attracted to susceptible plant roots because of a chemotropic response. Zentmeyer (1961) observed that in P. cinnamomi the substance was specific to living avocado roots. He also presented evidence that the substance showed host specificity, that is, avocado roots did not attract zoospores or germ tubes of P. citrophthora. Results obtained in present investigation suggest that invasion and pathogenesis of C. cucurbitarum to squash is related to a chemotropic response of germ tubes to floral tissues.

In the field, bees appeared with the opening of blossoms, but the spotted cucumber beetle was not observed until disease had been initiated in the field. Wolf (1917) observed that C. cucurbitarum was air-borne by isolating spores of Choanephora cucurbitarum on exposed culture dishes in the field; however, the possibility of insects contaminating plates was not mentioned by the author.

Wash suspensions and isolations on culture media indicate that bees and spotted cucumber beetles are instrumental in disseminating the pathogen. Appendaged sporangiospores were not found associated with bees or beetles but monoporous sporangiola were isolated from both.

The results of pathogenicity indicate that the fungus may enter through wounds (Palm and Jochems, 1924) or uninjured plant surfaces. Wolf (1917) observed the pathological anatomy of disease in fruit and corolla tissues of squash. He concluded that the fungus passes from the corolla into the fruit. The reports of Palm and Jochems (1924) and Wolf (1917) failed to demonstrate the site and mode of entry or the penetration phenomenon. Attempts in this study to demonstrate the mode and site of entry with scanning electron microscopy were not successful because of inefficient fixation procedures. However, all other preliminary evidence suggests that the areas around the floral tube are very stimulatory to the spores of Choanephora cucurbitarum.

This investigation on parasitism of C. cucurbitarum on squash in northern Florida concludes that: (a) Choanephora survives as a soil-borne pathogen, (b) the probable sources of initial inoculum are infested soils and plant

debris, (c) monosporous sporangiola appear to be the prime propagules involved in pathogenesis, (d) bees and spotted cucumber beetles can be agents of dispersal for secondary cycles of the pathogen, and (e) the site of entry centers around the corolla and appears to be enhanced by a strong chemotropic response of germ tubes to all female floral organs.

Chapter VI

Figures 82-87. Blossom blight and fruit rot of Choanephora cucurbitarum in the field.

Figure 82. 'Scallopini' female flower.

Figure 83. Bee visiting male squash flower.

Figure 84. Spores of Choanephora on male blossoms.

Figure 85. 'Scallopini' fruit infection.

Figure 86. 'Sundance' infected fruit (arrow).

Figure 87. Spores radiating from flower-end of fruit.


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Chapter VI

Figures 88-93. Sporulation of C. cucurbitarum on squash decoctions.

Figure 88. Stamen decoction. 400X

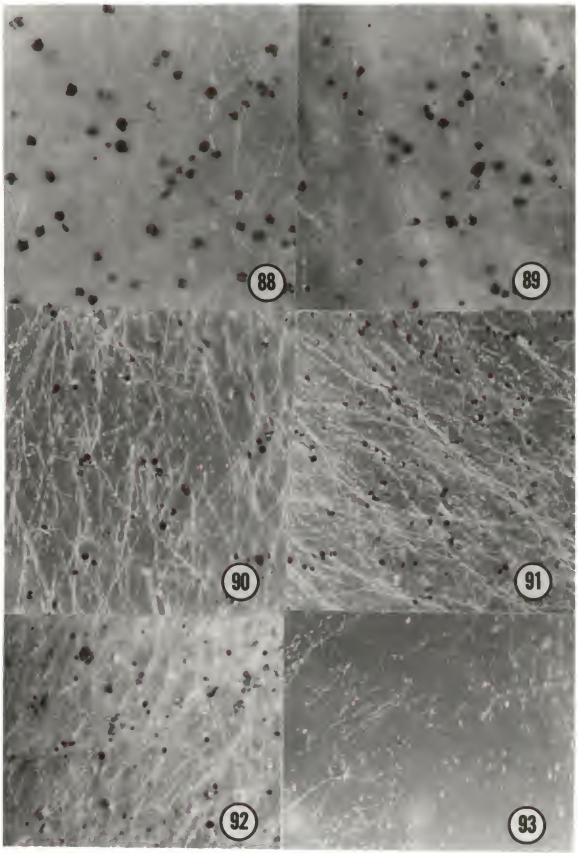
Figure 89. Pistils decoction. 400X

Figure 90. Male corolla decoction. 100X

Figure 91. Female corolla decoction. 100X

Figure 92. Fruit decoction. 100X

Figure 93. Water agar. 100X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Chapter VI

Figures 94-99 Chemotropic response of the germ tubes of C. cucurbitarum to squash tissues.

Figure 94. Stamen tissue, X100

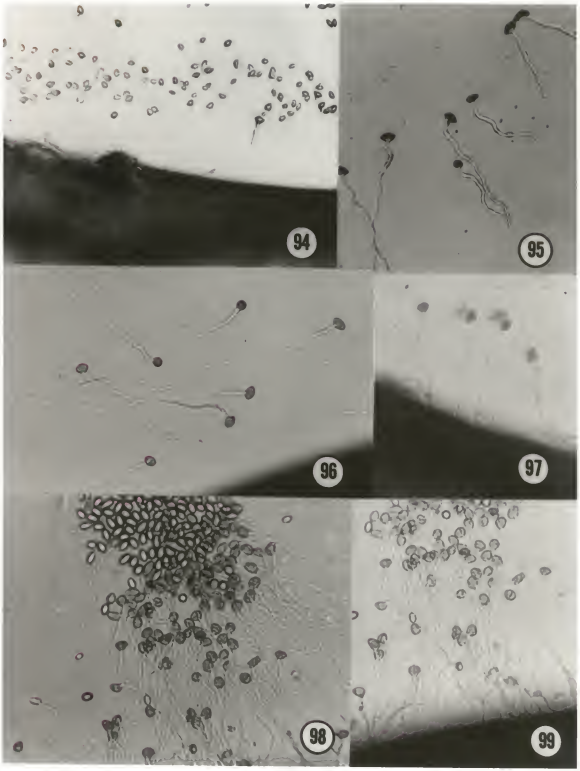
Figure 95. Stamen tissue, X200

Figure 96. Male corolla tissue. X200

Figure 97. Style tissue. X200

Figure 98. Female corolla tissue. X200

Figure 99. Fruit tissue. X200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BIOGRAPHICAL SKETCH

Grace Agee McWhorter was born January 15, 1948, in Mobile, Alabama. She received her high school diploma from Mobile County Training School, graduating as class salutatorian in May, 1966. She received her Bachelor of Science degree in Agriculture from Tuskegee Institute in 1970, graduating with high honors. In 1972, she received her Master of Science degree in Agriculture from Tuskegee Institute. In September 1974, she began part-time studies and full research responsibilities with Dr. James Kimbrough in the Department of Botany at the University of Florida. In 1975, she began full time doctoral studies in Botany, later transferring to the Department of Plant Pathology in December 1976. She held the position of visiting professor in Biology at the University of Missouri at St. Louis from August 1976 to June 1977. In June 1977, she returned to the University of Florida for further studies toward a doctorate in Plant Pathology.

Grace was joined in Holy Matrimony to Dr. George R. McWhorter on August 9, 1969. She is the mother of one

daughter, Kenya. Grace is the twelfth child born to Reverend and Mrs. Joe Agee. She has two brothers and nine sisters. She is a member of Delta Sigma Theta Sorority, the Mycological Society of America and the American Phytopathological Society. She has received both academic and social honors. She is a member of Beta Kappa Chi National Scientific Honor Society, nominee to Alpha Zeta Agricultural Honor Society, nominee for American Young Women of the Year Award, 1979, and was the recipient of the Florida Board of Regents State University System Grant for minorities in non-traditional areas of study.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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James W. Kimbrough, Chairman
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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